NPR1 Promotes Its Own and Target Gene Expression in Plant Defense by Recruiting CDK8

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NPR1 (NONEXPRESSER OF PR GENES1) functions as a master regulator of the plant hormone salicylic acid (SA) signaling and plays an essential role in plant immunity. In the nucleus, NPR1 interacts with transcription factors to induce the expression of NPR1 (NONEXPRESSER OF PR GENES1) functions as a master regulator of the plant hormone salicylic acid (SA) signaling and play a pivotal role in local and systemic immunity.

In nature, plants constantly face all kinds of abiotic and biotic stresses, including pathogens such as fungi, bacteria, oomycetes, nematodes, and viruses. Plants employ a battery of immune mechanisms that overcome infections caused by these pathogens (Jones and Dangl, 2006). The first layer of defense is called pathogen-associated molecular patterns (PAMP)-triggered immunity (PTI), in which plants utilize pattern recognition receptors to perceive conserved molecules in pathogens called PAMPs and induce defense responses against pathogen infection. However, many plant pathogens deliver PTI-suppressing effectors into plant cells, inducing effector-triggered susceptibility to cause diseases (Block et al., 2008). Facing these challenges, plants developed a second layer of defense, which involves Resistance proteins that recognize pathogen-specific effectors to mount effector-triggered immunity. Effector-triggered immunity is associated with a rapid and localized cell death called the hypersensitive response (Wu et al., 2014). Besides the induction of defense at the site of infection, a systemic defense response is often triggered in the distal parts of the infected plants, which protects uninfected tissues against subsequent infections by a wide range of pathogens (Mishina and Zeier, 2007; Fu and Dong, 2013). This long-lasting and broad-spectrum induced disease resistance is referred to as systemic...
acquired resistance (SAR; Pieterse et al., 2009). SAR is associated with increased levels of the plant hormone salicylic acid (SA) in plants (Shah, 2009; Vlot et al., 2009). Upon pathogen infection, an elevated level of SA activates the expression of PR (PATHOGENESIS-RELATED) genes, some of which encode proteins with antimicrobial activities (van Loon et al., 2006). Through genetic screens of Arabidopsis (Arabidopsis thaliana) mutants in which the expression of PR genes is abolished after treatment with SA or its active analogs, a locus called NPR1 (NONEXPRESSOR OF PR1) was identified (Cao et al., 1994; Ryals et al., 1997; Shah et al., 1997). The npr1 mutants, which are unable to activate the expression of PR genes, are more susceptible to virulent pathogen infection and are completely compromised in SAR (Cao et al., 1997). Later, NPR1 was identified as a receptor for SA by different groups (Wu et al., 2012; Ding et al., 2018). Even though it is known that NPR1 interacts with TGA and TCP transcription factors (TFs) in the nucleus to activate the expression of PR genes, including PR1, PR2, and PR5 (Després et al., 2000, 2003; Zhou et al., 2000; Kesarwani et al., 2007; Boyle et al., 2009; Lindermayr et al., 2010; Li et al., 2018), further studies are required to elucidate how NPR1-TF protein complexes promote the expression of PR genes.

Whereas posttranslational modifications of NPR1 protein including phosphorylation, S-nitrosylation, and sumoylation have been extensively studied (Tada et al., 2008; Xie et al., 2010; Lee et al., 2015; Saleh et al., 2015), transcriptional regulation of NPR1 is poorly understood. Yu et al. (2001) reported that the W-box sequence in the promoter of NPR1 gene is specifically recognized by the SA-induced WRKY18 protein in Arabidopsis. Mutations in the W-box sequence prevent WRKY18 from binding to the NPR1 promoter. A wild-type copy of the NPR1 gene with mutated W-box is unable to complement npr1 mutants and is unable to induce SA-dependent defense gene expression and disease resistance. This suggests that WRKY binding to the W-box sequence in the NPR1 promoter is essential for NPR1 gene expression. Interestingly, a number of WRKY genes were found to be induced by SA, which further suggests that these WRKYs could be involved in the regulation of SA-dependent responses (Wang et al., 2006). Indeed, it was found that WRKY6 protein can bind directly to the NPR1 promoter containing the W-box motif in a chromatin immunoprecipitation (ChIP) assay (Chai et al., 2014). The NPR1 mRNA level is reduced in wrky6 mutants and increased in WRKY6-overexpressing transgenic Arabidopsis in response to SA treatment (Chai et al., 2014).

In this study, we show that NPR1 protein positively regulates its own expression and binds to its own NPR1 promoter. Furthermore, we show that SA substantially promotes the interactions between NPR1 and CDK8 (CYCLIN-DEPENDENT KINASES) and between NPR1 and WRKY18. In cdk8 and CDK8-associated Mediator mutants, SAR is compromised and the expression of NPR1 and NPR1-dependent defense genes including PR1 is significantly reduced compared with wild-type plants. CDK8 interacts with WRKY6 and WRKY18, which are associated with the NPR1 promoter, to positively regulate the expression of NPR1. In addition, CDK8 interacts with both TGA5 and TGA7 and is associated with the PR1 promoter to regulate PR1 gene expression. Moreover, we found that CDK8 is involved in the recruitment of RNA polymerase II to the NPR1 and PR1 promoters and coding regions to promote the expression of these two genes. Taken together, our study uncovers a unique mechanism in which NPR1 recruits CDK8 and thereby promotes its own and target gene expression for the establishment of plant immunity.

RESULTS

NPR1 Protein Facilitates NPR1 Gene Expression

The npr1-1 and npr1-2 mutants were identified by screening for ethyl methanesulfonate (EMS)-induced mutants in which SA and its active analogs could not induce PR gene expression (Cao et al., 1997). The npr1-1 mutant carries a C1000-to-T mutation, changing the conserved His residue at position 334 in the third ankyrin-repeats sequence to a Tyr (Cao et al., 1997), whereas the npr1-2 mutant has a G449-to-A mutation that changes Cys-150 to Tyr (Cao et al., 1997; Zhou et al., 2000; Fan and Dong, 2002). We are fascinated by the fact that a single nucleotide mutation, which results in a single amino acid change, completely abolishes the function of NPR1 in plant defense. The first question we asked was whether any npr1-1 or npr1-2 transcript or npr1-1 or npr1-2 protein was present in these mutants (Cao et al., 1997; Spoel et al., 2009).

To address this question, we first checked NPR1 gene expression in Columbia-0 (Col-0) wild-type and npr1-1 and npr1-2 mutant plants. Interestingly, we found that both mutants accumulated significantly lower amounts of NPR1 transcript compared with Col-0 after SA treatment (Fig. 1A). To determine npr1 protein levels in npr1-1 and npr1-2 mutants, we first checked if an anti-NPR1 antibody can detect the npr1-1 and npr1-2 protein expressed in yeast (Saccharomyces cerevisiae) cells. As shown in Supplemental Figure S1, distinct bands were observed using lysate from yeast cells expressing npr1-1 or npr1-2, but not using the lysate of control yeast cells with an empty vector, demonstrating that this anti-NPR1 antibody detects npr1-1 and npr1-2 proteins efficiently (Supplemental Fig. S1). To test whether npr1-1 and npr1-2 mutants are indeed null mutants, we used this anti-NPR1 antibody to detect npr1-1 and npr1-2 protein levels in npr1-1 and npr1-2 mutants. As shown in Figure 1B, after SA treatment, a strong NPR1 band was detected in wild-type Col-0 plants; however, in npr1-1 and npr1-2 mutants, no npr1 protein was detected. It has been shown that NPR1-green fluorescent protein (GFP) expressed under the control of the 355 promoter complements npr1-2 mutants (Spoel et al., 2009). When we checked the NPR1-GFP protein level in...
35S:NPR1-GFP transgenic plants in the npr1-2 background using this anti-NPR1 antibody, we also detected a strong npr1-2 band that was absent in the npr1-2 mutant, indicating that a functional NPR1 protein is required for the SA induction of NPR1 protein (Supplemental Fig. S2). To exclude the possibility that the npr1-2 band was due to degradation of NPR1-GFP protein, we ran a separate gel using the same samples and probed the blot with an anti-GFP antibody. As shown in Supplemental Figure S2, we observed a strong NPR1-GFP band but no GFP band in the 35S:NPR1-GFP npr1-2 plants, suggesting that the npr1-2 band in 35S:NPR1-GFP npr1-2 plants was not due to degradation of NPR1-GFP protein.

These data prompt us to speculate that a functional NPR1 protein promotes NPR1 gene expression. To further demonstrate that NPR1 promotes its own expression, we designed a pair of specific primers that amplify the npr1-2 coding region sequence from 429 to 541 with an additional T448-to-G mutation in the forward primer. In this case, there is only a nucleotide change in the forward primer compared with the npr1-2 complementary DNA (cDNA) sequence, but two nucleotides are altered compared with wild-type NPR1 cDNA, so this pair of primers can amplify npr1-2 but not wild-type NPR1 cDNA. As shown in Figure 1C, the npr1-2 transcript was detected in npr1-2 mutants but not in Col-0 wild-type plants. Intriguingly, we observed a strong induction of npr1-2 mRNA in 35S:NPR1-GFP npr1-2 plants compared with npr1-2 mutants after they were treated with SA, indicating that NPR1-GFP promotes npr1-2 expression.

NPR1 Binds to Its Own Promoter

As a transcriptional coactivator, NPR1 binds to the promoters of the PR gene to facilitate their expression (Saleh et al., 2015). Based on these observations, we hypothesized that NPR1 protein positively regulates its own expression by binding to its own promoter. To demonstrate that NPR1 indeed binds to its own promoter, a ChIP assay was performed using 35S:GFP transgenic plants and 35S:NPR1-GFP transgenic plants in the npr1-2 background treated with 0.5 mM SA for 4 h.

Figure 1. NPR1 protein facilitates its own expression and binds to its own promoter. A, NPR1 gene expression level in Col-0 wild type, npr1-1, and npr1-2. The plants were sprayed with 0.5 mM SA or water as a control for 4 h. Leaves were collected for gene expression analysis. B, NPR1 protein level in Col-0 wild type, npr1-1, and npr1-2. Plants were treated as in A. Western blots were probed with an anti-NPR1 antibody. C, npr1-2 gene expression level in Col-0 wild type, npr1-2, and NPR1-GFP npr1-2. Seedlings were treated with 0.5 mM SA or water as a control for 4 h. Samples were assayed for gene expression analysis with primers specifically recognizing npr1-2 but not NPR1. The expression levels of NPR1 and npr1-2 were normalized to UBQ5 expression. ND, Not detected. D, NPR1 associates with its own promoter. Seedlings of transgenic GFP npr1-2 and NPR1-GFP npr1-2 were treated with 0.5 mM SA for 4 h. ChIP assay was performed using GFP-Trap (ChromoTek) or control beads with the following primers: -2000bp, amplifies upstream of the NPR1 promoter at the −2,000-bp region; W box, amplifies the sequence containing the W-box at the NPR1 promoter; Actin2 was used as a negative control. All experiments were repeated at least two times with similar results. Data represent means of three independent samples with s. Asterisks indicate significant differences (Student’s t test, *, P < 0.05). Ponceau S, Ponceau staining.
Interestingly, we found that the NPR1-GFP association with the W-box of the NPR1 promoter was enriched twofold compared with the GFP control (Fig. 1D). By contrast, no difference was found when the Actin2 gene was used as a negative control. Taken together, these data indicate that NPR1 protein positively regulates NPR1 gene expression and is associated with its own promoter.

The Interaction between NPR1 and WRKY18 Is Enhanced by SA

To investigate how NPR1 is associated with its own promoter, we used yeast two-hybrid (Y2H) assays to test the interaction between NPR1 and WRKY6 and WRKY18, which are TFs that are known to bind the NPR1 promoter (Yu et al., 2001; Chai et al., 2014). The yeast strain AH109 cotransformed with pGBK17-WRKY18 and pGADT7-NPR1, but not pGADT7-npr1-1 or -npr1-2, grew on the triple synthetic dropout (TD; −Leu, −Trp, and −His) plate, indicating that NPR1 but not npr1-1 or npr1-2 interacts with WRKY18 (Fig. 2A). Interestingly, the growth of yeast AH109 cotransformed with pGADT7-NPR1 and pGBK17-WRKY18 increased on the TD plate supplemented with 200 μM SA, indicating that SA promotes the interaction between NPR1 and WRKY18. No interaction between WRKY6 and NPR1 was found in Y2H assays (Fig. 2A). To further confirm the interaction between NPR1 and WRKY18, we performed a coimmunoprecipitation (Co-IP) assay in which GFP, WRKY6-GFP, or WRKY18-GFP and human influenza hemagglutinin (HA)-NPR1 were coexpressed in Nicotiana benthamiana leaves. We found that HA-NPR1 coimmunoprecipitated with WRKY18-GFP but not with WRKY6-GFP, indicating that WRKY18 but not WRKY6 interacts with NPR1 in planta (Fig. 2B). To further demonstrate the effect of SA on the interaction between NPR1 and WRKY18, we performed an in vitro pull-down assay. N. benthamiana leaves were used to transiently express GFP or WRKY18-GFP by agroinfiltration. GFP or WRKY18-GFP was then immunoprecipitated from leaf extracts by GFP-Trap. The beads were washed and

![Figure 2](https://www.plantphysiol.org/)

**Figure 2.** The interaction between NPR1 and WRKY18 is substantially enhanced by SA. A, NPR1 but not npr1-1 or npr1-2 interacts with WRKY18 in Y2H assays. Yeast strain AH109 was cotransformed with pGBK17-WRKY6 or WRKY18 and pGADT7-NPR1, npr1-1, npr1-2, or an empty vector (EV). The yeast strain AH109 cotransformed with pGBK17-WRKY18 and pGADT7-NPR1, npr1-1, npr1-2, or an empty vector (EV) was recultured, diluted to OD600 nm = 1.0, 0.1, or 0.01, and then 10 μL of the diluted liquid cultures was placed onto a DD plate and a TD plate (−Leu, −Trp, −His) with or without 200 μM SA. B, Co-IP between HA-NPR1 and GFP, WRKY6-GFP, or WRKY18-GFP proteins, expressed in N. benthamiana leaves by agroinfiltration, were immunoprecipitated with GFP-Trap for 2 h. The beads were washed, eluted with 2× loading buffer, and immunoblotted with an anti-GFP or an anti-HA antibody. C, In vitro pull-down between 6xHis-MBP-NPR1 and WRKY18-GFP. GFP or WRKY18-GFP was immunoprecipitated from N. benthamiana leaf extracts with GFP-Trap. The beads were washed and incubated with purified 6xHis-MBP-NPR1 with or without 200 μM SA. After overnight incubation, beads were washed three times with washing buffer, then eluted, and detected with anti-GFP or anti-6xHis antibody. The number beneath each blot indicates the relative strength of the band. The experiments were repeated two times with similar results.
incubated with 1 µg of 6xHis-Maltose Binding Protein (MBP)-NPR1 with or without 200 µM SA overnight. We found that 6xHis-MBP-NPR1 was pulled down by WRKY18-GFP but not by the GFP control and substantially more 6xHis-MBP-NPR1 protein was pulled down by WRKY18-GFP in the presence of SA than in its absence (Fig. 2C), indicating that SA promotes the interaction between WRKY18 and NPR1.

**SA Promotes the Interaction between NPR1 and CDK8**

NPR1 controls the expression of over 2,000 genes (Wang et al., 2006). Being a transcriptional coactivator, NPR1 may play a role in communicating with RNA polymerase II to promote its own expression; however, we did not observe any direct interaction between NPR1 and RNA polymerase II C-terminal domain (CTD) in our Y2H assays (data not shown). Since CDK8 phosphorylates RNA polymerase II CTD (Wang and Chen, 2004), we hypothesize that CDK8 could be the link between NPR1 and RNA polymerase II. To determine whether CDK8 interacts with NPR1, we performed Y2H assays using NPR1 as the bait and CDK8 as the prey. We found that NPR1 but not npr1-1 or npr1-2 interacts with CDK8 strongly on the TD plate. To determine whether SA can enhance this interaction, we tested the yeast growth on the TD plate supplemented with 1 mM 3-aminotriazole (3-AT) or 1 mM 3-AT and 200 µM SA. Indeed, SA dramatically promoted the yeast growth on the TD plates supplemented with 1 mM 3-AT (Fig. 3A), indicating that SA facilitates the interaction between NPR1 and CDK8 in Y2H assays. To further confirm the interaction between NPR1 and CDK8, a glutathione S-transferase (GST) pull-down assay was performed. We found that 6xHis-MBP-CDK8 was pulled down by GST-NPR1 but not by the GST control (Fig. 3B). In the presence of SA, more CDK8 protein was pulled down by GST-NPR1, indicating that SA promotes the direct interaction between NPR1 and CDK8 in vitro. To determine whether CDK8 and NPR1 expressed in plants interact with each other, a Co-IP assay was performed. GFP or NPR1-GFP and HA-CDK8 were coexpressed in N. benthamiana NahG transgenic plants. NahG encodes SA hydroxylase, which degrades SA into catechol (Delaney et al., 1994).

![Figure 3](image-url). **Figure 3.** SA promotes the interaction between NPR1 and CDK8. A, NPR1 but not npr1-1 or npr1-2 interacts with CDK8 in Y2H assays. Y2H assays were carried out using CDK8 as bait (in pGBK7 vector with a DNA-binding domain) and NPR1, npr1-1, npr1-2, or an EV as prey (in pGADT7 vector with an activation domain). Cotransformed yeast selected from a DD plate were recultured, diluted to OD_{600 nm} = 1, 0.1, or 0.01, and then 10 µL of the diluted liquid cultures was placed onto a DD plate and a TD plate with or without 1 mM 3-AT or 200 µM SA. B, In vitro pull-down assay between GST-NPR1 and 6xHis-MBP-CDK8. Purified 6xHis-MBP-CDK8 was incubated with magnetic beads conjugated with GST or GST-NPR1 with or without 200 µM SA. After overnight incubation, beads were washed four times with washing buffer, then eluted, and detected with anti-GST or anti-6xHis antibody. C, Co-IP between HA-CDK8 and NPR1-GFP proteins in N. benthamiana NahG transgenic plants. Proteins, extracted from N. benthamiana leaves transiently expressing NPR1-GFP and HA-CDK8 or GFP and HA-CDK8 proteins by agroinfiltration, were immunoprecipitated with GFP-Trap with or without 200 µM INA overnight. The beads were washed, eluted with 2× loading buffer, and immunoblotted with an anti-GFP or an anti-HA antibody. The number beneath each blot indicates the relative strength of the band. The experiments were repeated two times with similar results.
We use NahG transgenic plants to eliminate SA induced by agroinfiltration in N. benthamiana plants. We found that HA-CDK8 was communoprecipitated with NPR1-GFP but not with the GFP control. To test the effect of SA on the interaction between NPR1 and CDK8, we added 2,6-dichloroisonicotinic acid (INA), an active SA analog that cannot be degraded by NahG (Clarke et al., 1998; Fu et al., 2012; Li et al., 2017; Qi et al., 2018), in the NahG plant extract during the immunoprecipitation step. We found that INA strongly increased the amount of HA-CDK8 that was communoprecipitated (Fig. 3C), supporting that INA promotes the interaction between NPR1 and CDK8.

**CDK8 Plays an Important Role in Plant Immunity**

To determine whether CDK8 plays a role in plant immunity, we first checked the expression of CDK8 in Arabidopsis Col-0 plants after they were infected by *Pseudomonas syringae* pv *maculicola* ES4326 (*Psm* 4326) carrying the avirulent gene *avrRpt2*, which is a trigger for SAR. We found that *Psm avrRpt2* infection induced CDK8 gene expression slightly at 4 h and significantly \((P < 0.05)\) by 3.3- and 3.4-fold at 8 and 12 h post inoculation, respectively (Fig. 4A). We also observed an even higher level of CDK8 gene expression at 24 h after inoculation (Fig. 4A). These data suggest that CDK8 plays a role in plant immunity. To determine whether CDK8 indeed plays a role in SAR, we performed a SAR assay in Col-0 wild-type, *npr1-2*, *cdk8-4*, and *cdk8-1* mutant plants. We found that both *cdk8-4* and *cdk8-1* mutants showed increased susceptibility compared with that of Col-0 in the absence of SAR induction (Fig. 4B). In terms of SAR, unlike in Col-0 plants, the growth of the virulent *Psm* 4326 on systemic leaves of *npr1-2*, *cdk8-4*, and *cdk8-1* mutants was not significantly reduced, even after preinoculation with the avirulent *Psm avrRpt2*, indicating that *cdk8-4* and *cdk8-1* mutants were SAR defective (Fig. 4B).

To investigate how CDK8 contributes to the establishment of SAR, we analyzed *NPR1* gene expression in the local leaves of Col-0, *npr1-2*, and *cdk8-4* mutants 4 h after infection with *Psm avrRpt2*, because NPR1 is a master regulator of local and systemic plant immunity (Cao et al., 1997). We found that the *NPR1* gene was dramatically induced by *Psm avrRpt2* in Col-0. Interestingly, the expression of the *NPR1* gene was significantly \((P < 0.05)\) reduced in *npr1-2* and *cdk8-4* mutants compared with Col-0 (Fig. 4C). We next analyzed the expression of *PR1*, a widely used marker gene of SAR, in the local leaves of Col-0, *npr1-2*, and *cdk8-4* mutants after 24 h of infection with *Psm avrRpt2*. We found that *PR1* gene expression was dramatically reduced in the *cdk8-4* mutant compared with Col-0 (Fig. 4D). Similarly, we also observed a reduction of *PR1* gene expression in the local leaves of the *cdk8-4* mutant compared with Col-0 when challenged with virulent *Psm* (Supplemental Fig. S3). In addition, we found that *PR1* gene expression was drastically reduced in *npr1-2* and *cdk8-4* mutant systemic leaves when compared with Col-0 after 2 d of infection with *Psm avrRpt2* (Fig. 4E). Taken together, these results indicate that CDK8 plays an important role in plant immunity.

**The Expression of NPR1 and Its Target Genes Including PR1 Requires NPR1 and CDK8 in SA Signaling**

To further validate the function of CDK8 in SA signaling, we irrigated soil-grown Col-0 and *npr1-2* and *cdk8-4* mutant plants with 0.5 mM SA, then analyzed *NPR1* gene expression after 4 h of treatment. We found that SA could induce *NPR1* gene expression in the *cdk8-4* mutant, but the level was significantly \((P < 0.01)\) lower than that in Col-0 (Fig. 4F). Meanwhile, we also checked *NPR1* gene expression in these same genotypes 4 h after they were sprayed with SA. Consistent with the results shown in Figure 4F, we observed significantly reduced *NPR1* gene expression in *cdk8-4* and *npr1-2* mutants compared with Col-0 (Supplemental Fig. S4A). To determine the *NPR1* protein level in *npr1-2* and *cdk8-4* mutants, we performed immunoblot assays using Col-0 wild-type and *npr1-2* and *cdk8-4* mutant plants subjected to SA treatment. We found that both *npr1-2* and *cdk8-4* mutants accumulated much less *NPR1* protein compared with that in Col-0 (Fig. 4G). In the *npr1-2* mutant, SA treatment causes the cotyledons to appear yellow due to overaccumulation of SA (Zhang et al., 2010). We reasoned that the *cdk8-4* mutant would also show this phenotype, because *NPR1* gene expression is reduced in these mutant plants. To test this, we placed *cdk8-4* seeds on Murashige and Skoog plates containing 0.35 mM SA and then recorded the ratio of seedlings showing yellow cotyledons after 7 d. Surprisingly, we found that 95% of *cdk8-4* seedlings developed yellow cotyledons on SA plates, which is very close to the 100% yellow cotyledon phenotype observed in *npr1-2* mutants (Supplemental Fig. S4B). We next checked the expression of *NPR1*-dependent genes in these mutants and found that the expression of *PR1*, *PR2*, and *WRKY38* in the *cdk8-4* mutant was dramatically reduced compared with Col-0 (Fig. 4, H–J). Together, these data indicate that CDK8 is required for the full expression of *NPR1* and its target genes including *PR1* in SA signaling.

**CDK8 Regulates NPR1 Protein Accumulation and PR1 Gene Expression in a Kinase-Independent Manner**

In mammals, yeast, and Arabidopsis, CDK8 phosphorylates the RNA polymerase II CTD (Liao et al., 1995; Wang and Chen, 2004). We wondered if CDK8 regulates *NPR1* and *PR1* gene expression in a kinase-dependent manner. To test this, we treated Col-0, *cdk8-1*, CDK8-MYC *cdk8-1*, and kinase-dead CDK8 (CDK8KD-MYC) *cdk8-1* plants with 0.5 mM SA to detect *NPR1* protein level and *PR1* gene expression (Zhu et al., 2014). As we expected, *cdk8-1*, like *cdk8-4*, accumulated a substantially lower level of *NPR1* protein and exhibited
Figure 4. SAR and defense gene expression are compromised in the cdk8 mutants. A, CDK8 gene expression level during the time-course infection of the avirulent pathogen. Col-0 plants were infiltrated with Psm avrRpt2 (OD_{600 nm} = 0.005) or MgCl_{2} buffer as a control. Leaf samples were collected at the indicated times for gene expression analysis. hpi, Hours post inoculation. B, SAR phenotypes in Col-0 wild-type, npr1-2, cdk8-4, and cdk8-1 mutant plants. Two lower leaves were infiltrated with Psm avrRpt2 (OD_{600 nm} = 0.02; SAR +) or MgCl_{2} buffer (SAR −) as a control. After 2 d, two upper healthy leaves were challenged with virulent Psm (OD_{600 nm} = 0.0005). The leaf discs from the second inoculation were collected at 3 d post inoculation for counting bacterial colonies. CFU, Colony-forming unit. C, NPR1 gene expression levels in the local leaves of Col-0 wild-type, npr1-2, and cdk8-4 mutant plants infected with Psm avrRpt2 (OD_{600 nm} = 0.02) or MgCl_{2} buffer as a control. Leaves were collected 4 h after infection for gene expression analysis. D, PR1 gene expression level in the leaves of Col-0 wild-type, npr1-2, and cdk8-4 mutant plants. Leaves were infected with Psm avrRpt2 (OD_{600 nm} = 0.001) or MgCl_{2} buffer as a control. After 24 h, the infected leaves were collected for gene expression analysis. E, PR1 gene expression levels in the systemic leaves of Col-0 wild-type, npr1-2, and cdk8-4 mutant plants. Two lower leaves were infected with Psm avrRpt2 (OD_{600 nm} = 0.02) or MgCl_{2} buffer as a control. After 2 d, the upper uninfected leaves were collected for gene expression analysis. F, NPR1 protein accumulation in Col-0 wild type, npr1-2, and cdk8-4 mutants during SA treatment. H to J, Col-0 wild type, npr1-2, and cdk8-4 mutants were treated with SA for 24 h, and leaf samples were collected for PR1 (H), PR2 (I), and WRKY38 (J) gene expression analysis. The expression levels of CDK8, NPR1, PR1, PR2, and WRKY38 were normalized to UBQ5 expression. Data represent means of three independent samples with SE. Asterisks indicate significant differences (Student’s t test, *, P < 0.05 and **, P < 0.01). The experiments were repeated three times with similar results.
reduced PR1 gene expression compared with that in Col-0 (Supplemental Fig. S5A). CDK8-MYC cdk8-1 showed a similar level of NPR1 protein level and PR1 gene expression compared with Col-0 (Supplemental Fig. S5B), indicating that CDK8-MYC is functional and able to complement the cdk8-1 phenotype. Surprisingly, we found that both the NPR1 protein level and PR1 gene expression in CDK8(KD)-MYC cdk8-1 plants are also higher than in the cdk8-1 mutant (Supplemental Fig. S5B), indicating that CDK8 regulates NPR1 protein accumulation and PR1 gene expression in a kinase-independent manner.

CDK8-Associated Mediator12 and Mediator13 Positively Regulate the Expression of SAR and Defense Genes

Besides phosphorylating CTD of RNA polymerase II, another major function of CDK8 is that it forms the kinase module of the Mediator complex with cyclin C, Mediator12, and Mediator13 (Jeronimo and Robert, 2017). Since we found that CDK8 regulates NPR1 and PR1 gene expression in a kinase-independent manner, we hypothesize that it functions together with its associated subunits in the kinase module of the Mediator complex, so we analyzed the SAR phenotype in Col-0, npr1-2, cycCab (a single T-DNA insertion line in which both CycCa and CycCb genes are down-regulated), med12, and med13 (subunits of the CDK8 kinase module in the Mediator complex) mutants. We found that both med12 and med13 mutants showed significantly (P < 0.05) higher susceptibility compared with Col-0, whereas the cycCab mutant showed modestly increased bacterial growth (Fig. 5A). In terms of SAR, the mediator mutants showed completely compromised SAR. In the cycCab mutant, the Psm growth was significantly reduced by 5-fold after SAR induction, indicating that SAR is not defective in the cycCab

Figure 5. CDK8-associated Mediators are involved in SAR and defense gene expression. A, SAR phenotypes in Col-0 wild-type, npr1-2, cycCab, med12, and med13 mutant plants. Two lower leaves were infiltrated with Psm avrRpt2 (OD600 nm = 0.02; SAR +) or MgCl2 buffer (SAR −) as a control. After 2 d, two upper healthy leaves were challenged with virulent Psm (OD600 nm = 0.0005). The leaf discs from the second inoculation were collected at 3 d post infection for counting bacterial colonies. CFU, Colony-forming units. B, PR1 gene expression levels in the systemic leaves of Col-0 wild-type, npr1-2, cycCab, med12, and med13 mutant plants. Two lower leaves were infected with Psm avrRpt2 (OD600 nm = 0.02) or MgCl2 buffer as a control. After 2 d, the upper uninfected leaves were collected for PR1 gene expression analysis. C and D, NPR1 and PR1 gene expression levels in Col-0 wild-type, npr1-2, cycCab, med12, and med13 mutant plants treated with SA. Col-0 and mutant plants were irrigated with 0.5 mM SA, and then leaf samples were collected after 4 h for NPR1 gene expression analysis (C). After 24 h, leaves were collected for PR1 gene expression analysis (D). The expression levels of NPR1 and PR1 were normalized to UBQ5 expression. Data represent means of three independent samples with se. Asterisks indicate significant differences (Student’s t test, * P < 0.05). Columns with different letters indicate significant differences determined by Duncan’s multiple range test. The experiments were repeated three times with similar results.
mutant (Fig. 5A). This could be because both *CycCa* and *CycCb* genes are only partially knocked down in this single T-DNA insertion line, based on a previous report (Zhu et al., 2014).

We next analyzed the expression of *PR1*, a widely used marker gene of SAR, in the systemic leaves of Col-0 and *npr1-2*, *med12*, and *med13* mutants after 48 h infection of *Psm avrRpt2* within local leaves. We found that *PR1* gene expression was also dramatically reduced in *med12* and *med13* mutants but only slightly, but not significantly, reduced in the *cycCab* mutant compared with Col-0 (Fig. 5B). To determine whether *CycCa*, *CycCb*, *MED12*, and *MED13* are involved in SA signaling, we analyzed the *NPR1* and *PR1* gene expression levels in these mutants 4 and 24 h after SA irrigation. Interestingly, we found that the expression levels of both *NPR1* and *PR1* genes in *med12* and *med13* mutants were extremely low, similar to the *npr1-2* mutant, indicating that both *MED12* and *MED13* play important roles in *NPR1* and *PR1* gene expression (Fig. 5, C and D).

**CDK8 Interacts with WRKY6 and WRKY18 and Is Required for RNA Polymerase II to Bind the Promoter and Coding Region of NPR1**

Recently, several studies have shown that the Mediator complex plays an important role in plant defense gene expression, but the underlying mechanism has not been fully elucidated (Canet et al., 2012; Zhang et al., 2012, 2013; An and Mou, 2013; Wang et al., 2015, 2016). Mediator regulates gene expression by acting as a bridge between TFs and RNA polymerase II, which catalyzes the transcription of DNA to synthesize pre-cursors of mRNA and most microRNAs (Sims et al., 2004).

Because Mediator cannot directly interact with DNA (Uthe et al., 2017), it must be associated with TFs to regulate gene expression in response to different stimuli (Yang et al., 2016; Jeronimo and Robert, 2017). It has been shown that SRB10/CDK8 interacts with many TFs in yeast and mammalian cells (Galbraith et al., 2010; Nemet et al., 2014). In addition, CDK8 phosphorylates RNA polymerase II at the CTD domain in Arabidopsis (Wang and Chen, 2004). In previous studies, it was demonstrated that WRKY6 and WRKY18 bind to the *NPR1* promoter and that this binding is required for *NPR1* gene expression induced by SA (Chai et al., 2014). Furthermore, we found that CDK8 positively regulates *NPR1* gene expression in response to SA (Fig. 4F; Supplemental Fig. S4). Therefore, we hypothesize that CDK8 regulates *NPR1* gene expression through its association with WRKY6 and/or WRKY18. To test this, we performed Y2H assays using CDK8 as the bait and WRKY6, WRKY18, and WRKY36 as prey. Interestingly, we found that CDK8 interacted with WRKY6 and WRKY18 but not with WRKY36 in Y2H assays (Fig. 6A). To confirm this interaction in planta, we performed a Co-IP assay in *N. benthamiana*. Agroinfiltration was used to transiently coexpress GFP, WRKY6-GFP, WRKY18-GFP, and WRKY36-GFP proteins with HA-CDK8 in *N. benthamiana*. We found that HA-CDK8 coimmunoprecipitated with WRKY6-GFP and WRKY18-GFP proteins (Fig. 6B). In contrast, HA-CDK8 did not coimmunoprecipitate with GFP or WRKY36-GFP control protein, confirming that WRKY6 and WRKY18 interact with CDK8 in planta. Taken together, these data indicate that CDK8 interacts with WRKY6 and WRKY18.

Since WRKY6 and WRKY18 can bind to the *NPR1* promoter to regulate *NPR1* gene expression (Yu et al., 2001; Chai et al., 2014), we speculated that CDK8 regulates *NPR1* gene expression by its association with the *NPR1* promoter. To test this, we performed ChiP assays using Col-0 and CDK8-MYC cdk8-1 transgenic plants to determine the association of CDK8 with the target gene. Indeed, we found that the CDK8-MYC association with the W-box region was significantly (*P < 0.05*) enriched compared with that in Col-0 (Fig. 6C). By contrast, no difference was found when Actin2 gene was used as a negative control (Fig. 6C). Collectively, these data indicate that CDK8 interacts with WRKY6 and WRKY18 proteins and regulates *NPR1* gene expression through its association with the *NPR1* promoter.

To further elucidate how CDK8 regulates *NPR1* gene expression, we tested if CDK8 is required for RNA polymerase II binding to the *NPR1* promoter using ChiP assays. We found that RNA polymerase II enrichment on the W-box of the *NPR1* promoter in the cdk8-4 mutant was significantly (*P < 0.05*) reduced compared with Col-0 wild-type plants (Fig. 6D), indicating that CDK8 is involved in RNA polymerase II binding to the *NPR1* promoter at the W-box motif. Interestingly, we also found that RNA polymerase II enrichment on the *NPR1* gene-coding region in the cdk8-4 mutants was significantly (*P < 0.05*) reduced compared with Col-0 wild-type plants (Fig. 6D), indicating that CDK8 is involved in RNA polymerase II binding to the coding region of *NPR1*. As a negative control, RNA polymerase II enrichment in the coding region of *Actin2* showed no difference between Col-0 and the cdk8-4 mutant (Fig. 6D). Taken together, these results suggest that CDK8 forms a molecular bridge between WRKY6/WRKY18 and RNA polymerase II to facilitate *NPR1* gene expression.

**CDK8 Interacts with TGAs and Is Associated with the PR1 Promoter**

It is well known that *PR1* gene expression is regulated by TGA TFs and the transcriptional coactivator *NPR1* in Arabidopsis (Després et al., 2000; Zhou et al., 2000; Kim and Delaney, 2002; Boyle et al., 2009); however, it is not clear how precisely *NPR1* coordinates with other transcription machinery to regulate *PR1* gene expression. We postulate that *NPR1* regulates *PR1*...
gene expression through its interaction with CDK8 and TGA TFs. Although we have already found that NPR1 gene expression is down-regulated in the cdk8-4 mutant, it is still essential to understand how CDK8 coordinates with TFs and other coactivators to regulate PR1 gene expression.

We screened seven TGA TFs to identify which TGA TFs interact with CDK8 in Y2H assays using CDK8 as the bait and TGAs as the prey (Després et al., 2000; Zhou et al., 2000; Kim and Delaney, 2002; Boyle et al., 2009). Our data show that, among seven TGAs, only TGA5 and TGA7 were found to interact with CDK8 specifically in Y2H assays (Fig. 7A), whereas TGA1 showed very weak interaction with CDK8. To confirm the interactions between CDK8 and TGA5/TGA7, we performed a Co-IP assay using N. benthamiana leaves coexpressing GFP, TGA5-GFP, or TGA7-GFP with HA-CDK8. We found that HA-CDK8 communoprecipitated with TGA5-GFP and TGA7-GFP but not with the GFP control (Fig. 7B), confirming that TGA5 and TGA7 interact with CDK8 in planta.

It has been shown that NPR1 monomers interact with TGAs, which target the activation sequence-1 (as-1) element of the PR1 promoter (Zhou et al., 2000). We hypothesized that CDK8 is associated with the PR1 promoter and coding region in ChIP assays. Col-0 and cdk8-MYC cdk8-1 transgenic plants were treated with 0.5 mM SA for 4 h. Chromatin was extracted, then immunoprecipitated with anti-MYC antibody. CDK8 is required for RNA polymerase II (Pol II) association with the PR1 promoter and coding region in ChIP assays. Col-0 and cdk8-4 plants were treated with 0.5 mM SA for 16 h. Chromatin was extracted, then immunoprecipitated with anti-RNA polymerase II antibody or IgG as a negative control. Primers used in the quantitative PCR (qPCR; C and D) amplify the following amplicons: -2000bp, amplifies upstream of the PR1 promoter at the -2,000-bp region; W-box, amplifies the sequence containing the W-box at the PR1 promoter; Actin2 was used as a negative control. Data represent means of three independent samples with st. Asterisks indicate significant differences (Student's t test, *, P<0.05). The experiments were repeated at least two times with similar results.
assays. Interestingly, we also found RNA polymerase II enrichment on the as-1 sequence, and the coding region of PR1 was also significantly (P < 0.05) reduced in the cdk8-4 mutants compared with Col-0 wild-type plants (Fig. 7D), indicating that CDK8 is required for the enrichment of RNA polymerase II on the PR1 promoter and coding region. Taken together, these data demonstrate that CDK8 forms a protein complex with NPR1, TGA5, and TGA7 and acts as a molecular bridge between RNA polymerase II and the PR1 promoter and the coding region and thereby facilitates PR1 gene expression.

**DISCUSSION**

As the master regulator of SA-mediated plant immunity, NPR1 controls the expression of over 2,000 genes (Wang et al., 2006; Fu and Dong, 2013). To exert its function, NPR1 is reduced from an oligomeric state to a monomeric state and then translocated to the nucleus after the accumulation of SA induced by pathogen infection (Mou et al., 2003). Most studies on NPR1 have been focused on posttranslational modifications (Withers and Dong, 2016). NPR1 paralogs NPR3 and NPR4 both function as SA receptors and adaptors for Cullin3 E3 ligase that mediate the degradation of NPR1 and thereby maintain an optimal level of NPR1 protein during plant defense (Fu et al., 2012). Intriguingly, we showed that NPR1 transcripts in the npr1-1 and npr1-2 mutants were dramatically lower compared with wild-type plants (Fig. 1A). Consistent with these observations, Zhang et al. (2012) also showed that Pst DC3000-induced NPR1 transcript accumulation in npr1-3 mutants was also significantly reduced.

![Figure 7. CDK8 interacts with TGAs and is associated with the PR1 promoter.](image-url)
lower than that in Col-0. NPR1 transcript accumulation in the npr1 mutants was not induced in response to INA (Kinkema et al., 2000). Our data indicate that a functional NPR1 protein is required for the full expression of NPR1 (Fig. 8). In addition to npr1-1 and npr1-2 mutants, EMS-induced mutagenesis, which frequently causes a G-to-A mutation, has been widely used for genetic screens to identify loss-of-function mutants. Intriguingly, a single-nucleotide or a single-amino acid change causes the loss of function of many important genes, including FLAGELLIN SENSING2, ENHANCED DISEASE SUSCEPTIBILITY1, RESISTANCE TO PSEUDOMONAS SYRINGAE2, and AVRPPHB SUSCEPTIBLE3, etc. (Falk et al., 1999; Gómez-Gómez and Boller, 2000; Axtell et al., 2001; Nobuta et al., 2007). It is worthwhile to revisit these EMS single-nucleotide mutants to find out whether they are also protein null mutants. For NPR1, dozens of mutants have been identified in Arabidopsis as loss-of-function mutants (Cao et al., 1997; Canet et al., 2010). In addition to NPR1, ABA INSENSITIVE5 and Snail Family Transcriptional Repressor1 have also been shown to positively regulate their own expression by binding to their own promoters (Peiró et al., 2006; Xu et al., 2014). Therefore, our study on NPR1 could provide insights into the autoregulation of ABIs5 and Sanil1.

Only two TFs, WRKY18 and WRKY6, have been found to bind to the NPR1 promoter to date (Yu et al., 2001; Chai et al., 2014). We found that both of them interact with CDK8 (Fig. 6, A and B), which brings CDK8 to the W-box region of the NPR1 promoter (Fig. 6C) to form a transcription initiation complex. CDK8 is also required for NPR1 transcription elongation, as it was shown that knockout of CDK8 reduces the binding of RNA polymerase II to the coding region of NPR1 (Fig. 6D). In agreement with our data, a previous report has shown that CDK8 is required for RNA polymerase II binding to the coding region of PDF1.2 to facilitate its expression and confer resistance to that fungal pathogen Alternaria brassicicola (Zhu et al., 2014). Likewise, CDK8 is essential for RNA polymerase II elongation in mammalian cells as well (Galbraith et al., 2013). Together, our data provide evidence that CDK8 functions as a molecular bridge between WRKY6/WRKY18 and the RNA polymerase II transcription machinery (Fig. 8). Thus, our research filled an important knowledge gap between WRKY6/WRKY18 and RNA polymerase II-mediated NPR1 gene transcription. In mammals and yeast, it has been shown that CDK8/SRB10 phosphorylates many TFs, including Ste12, Gcn4, Msn2, Gal4, E2F1, STAT1, and Phd1 (Hirst et al., 1999; Chi et al., 2001; Nelson et al., 2003; Raithatha et al., 2012; Bancerek et al., 2013). Therefore, besides recruiting RNA polymerase II to the gene promoters, CDK8 may also be involved in the phosphorylation of WRKY6 and/or WRKY18 TFs to activate NPR1 gene expression, which warrants further investigation. Surprisingly, we found that NPR1 protein is associated with its own promoter at the W-box motif (Fig. 1D). NPR1 interacts with WRKY18 but not WRKY6 in Y2H assays. The interaction between NPR1 and WRKY18 is greatly enhanced by SA (Fig. 2, A and C).

**Figure 8.** Schematic model of the roles of NPR1 and CDK8 in the transcriptional regulation of NPR1 and PR1 genes. Left, Activation of NPR1 gene expression by NPR1 and CDK8. Pathogen infection induces the expression of the CDK8 gene. CDK8 interacts with the WRKY6 and WRKY18 TFs, which bind to the NPR1 gene promoter at the W-box motif. SA promotes the interactions between NPR1 and CDK8 and between NPR1 and WRKY18. CDK8 and other proteins in the kinase module of the Mediator complex, including MED12 and MED13, bring RNA polymerase II (Pol II) to the NPR1 gene promoter and coding region to promote its transcription. NPR1 mRNA is then exported to the cytosol for NPR1 protein synthesis. Right, CDK8 facilitates the expression of the PR1 gene. After CDK8 and NPR1 induce the transcription of the NPR1 gene, NPR1 protein is synthesized and reduced from oligomers to monomers upon pathogen infection, then NPR1 monomers enter the nucleus. In the nucleus, NPR1 forms a protein complex with CDK8, TGA5, and TGA7, which bind to the PR1 promoter at the TGA sequence. RNA polymerase II is then recruited to the promoter and coding region of the PR1 gene by CDK8, MED12, and MED13 to facilitate PR1 gene expression to activate plant defense.
Interestingly, we found that NPR1, but not npr1-1 or npr1-2, interacts with CDK8, which interacts with WRKY6 and WRKY18. Together, these data suggest that NPR1 recruits CDK8, CDK8-associated RNA polymerase II, and WRKY18 to the NPR1 promoter to activate its expression. In agreement with this, we found that npr1-1 and npr1-2 mutants, in which mutated npr1-1 and npr1-2 protein cannot interact with CDK8 to activate the expression of the N PRI gene, showed a significantly lower NPR1 transcript level than that in Col-0 (Fig. 1A).

In Arabidopsis, NPR1, as a transcriptional coactivator, interacts with TGA and TCP TFs to facilitate PR gene expression (Després et al., 2000; Zhou et al., 2000; Johnson et al., 2003; Li et al., 2018); however, how NPR1 activates PR1 gene expression is still not clear. There is a missing link between NPR1 and the transcription machinery. Our data suggest that CDK8 could be the link, as SA promotes the interaction between NPR1 and CDK8 (Fig. 3). In our screens, we found that CDK8 interacts specifically with TGA5 and TGA7 TFs (Fig. 7, A and B), which function as positive regulators of PR1 gene expression and plant immunity (Kim and Delaney, 2002; Kesarwani et al., 2007; Song et al., 2011). Furthermore, we showed that CDK8 binds to the 5′-1 sequence in the PR1 promoter (Fig. 7C). Taken together, these data indicate that NPR1 recruits TGA5, CDK8, and RNA polymerase II to the PR1 promoter for transcriptional activation. In addition, our data show that CDK8 is required for RNA polymerase II binding to the 5′-1 sequence in the PR1 promoter and the PR1 coding region (Fig. 7D). Collectively, our data demonstrate that CDK8 connects the TGA TFs and the coactivator NPR1 with RNA polymerase II to facilitate PR1 gene expression (Fig. 8). Therefore, our data reveal a fundamental mechanism of PR1 gene regulation. A previous study has already shown that SA induces NPR1 sumoylation (Saleh et al., 2015). Sumoylation prevents NPR1 interaction with WRKY70, which functions as a transcription repressor of PR1, and promotes NPR1 interaction with TGA3, to facilitate PR gene expression (Saleh et al., 2015). It would be interesting to test how NPR1 sumoylation affects its interactions with CDK8, WRKY18, and TCP8, TCP14, and TCP15 to regulate plant defense (Li et al., 2018).

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis (Arabidopsis thaliana) ecotype Col-0 was used as wild-type plants. The mutants cdk8-4 (GABI_564F11), cycCdb (SALK_034900C), md12 (SALK_108241), and md13 (SALK_018056), which have been described in previous studies (Wang and Chen, 2004; Ng et al., 2013; Zhu et al., 2014), were obtained from GABI-KAT or the Arabidopsis Biological Resource Center (Kleinboelting et al., 2012). Seeds of cdk8-1, cdk8-MYC, cycCdb-1, and cycCdb-MYC were previously described in Zhu et al. (2014). The insertions and homozygosity were confirmed by genomic DNA PCR using the primers listed in Supplemental Table S1. Plants were grown in a growth chamber with a cycle of 12 h of light/12 h of dark, 22°C/20°C day/night temperature, and 60% relative humidity. Bacterial titer in the upper or systemic leaves was measured 3 d post infection.

Plasmid Construction

CDK8, WRKY6, and WRKY18 were cloned from a Col-0 cDNA library and cloned to the pDONR207 vector by BP reaction according to the manufacturer’s instructions. To obtain C-terminal GFP tag fusion proteins, WRKY6, WRKY18, WRKY36, TAG5, and TAG7 were cloned into pG7F7WQ2 by LR reaction. CDK8 was subcloned into pEARLEYGATE201 to obtain HA-CDK8. WRKys and TGA5s were cloned into pGADT7 and CDK8 was cloned into pGBK717 for the Y2H assay. All the constructs were verified by sequencing.

Plant Infection and Treatment

Pseudomonas syringae pv maculicola strain ES4326 and Psm avrApt2 were grown at 28°C in King’s B medium containing the appropriate antibiotics (100 mg L−1 streptomycin and 10 mg L−1 tetracycline). Bacteria were pelleted, washed three times with 10 mM MgCl2, resuspended, and diluted in 10 mM MgCl2 to the desired concentration. To induce SAR, two fully expanded leaves of each plant were infiltrated (primary inoculation) with a suspension containing Psm avrApt2 at OD600 = 0.02. In parallel, control plants were similarly infiltrated with 10 mM MgCl2. Two days later, two upper uninfected systemic leaves were further infiltrated with the virulent Psm 4326 at OD600 = 0.0005. Bacterial titer in the upper or systemic leaves was measured 3 d post infection.

RNA Extraction and Reverse Transcription qPCR

Total RNA was isolated using the Trizol reagent (Invitrogen) according to the manufacturer’s instructions. RNA quality and quantity were determined using a spectrophotometer (Eppendorf). Genomic DNA was removed by DNase I (Thermo Fisher Scientific) treatment, and first-strand cDNA was synthesized using qScript cDNA SuperMix (Quanta Biosciences). qPCR was carried out using SYBR Green SuperMix (Quanta Biosciences). UBIQUITINS (UBQ5) was used as a control. Gene-specific primers were designed using Primer Premier 5 software, and their sequences are shown in Supplemental Table S1.

Western Blotting

Rosette leaves from 5-week-old plants or 14-d-old seedlings were treated with 0.5 mM SA at the indicated times. Samples were collected, frozen in liquid nitrogen, and homogenized in protein extraction buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.1% (v/v) Triton X-100, 0.2% (v/v) Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, and 1× protease inhibitor cocktail). Homogenates were centrifuged (13,000 g) for 15 min at 4°C twice. Proteins were then denatured at 70°C with 5× loading buffer supplemented with 500 mM dithiothreitol (DTT) for 15 min. Western blots were probed with an anti-GFP (Clontech) or anti-NPR1 antibody (a gift from Dr. Zhonglin Mou).

Y2H Assays

To test the interactions between CDK8 and WRKYs or NPR1, the Saccharomyces cerevisiae yeast strain AH109 was cotransformed with pGBK717-CDK8 and pGADT7-EV, NPR1, npr1-1, npr1-2, WRKY6, WRKY18, or WRK6 according to the Clontech yeast transformation protocol. To test the interactions between CDK8 and TGA5s, yeast strains AH109 and Y187 were transformed with pGADT7-EV, pGADT7-TGAs, and pGBK717-CDK8. Yeast strains were mated in Yeast Peptone Dextrose Adenine medium overnight, and diploid yeast colonies were selected on synthetic dextrose (SD)-Trp-Leu (DD) plates. Fresh single colonies were grown in the SD-Trp-Leu liquid medium overnight. The mated or cotransformed yeast cultures were diluted to OD600 nm = 1.0, or 0.01 with sterilized distilled water. Ten microliters of yeast suspension was spotted on SD-Trp-Leu and SD-Trp-Leu-His (TD) plates with or without SD-AT. The interactions and yeast growth were observed after 4 to 7 d at 30°C.
phenylethylbutylfluoride, 50 μM MG115, and protease inhibitor cocktail) overnight. Beads were then washed five times with washing buffer (100 mM Tris-HCl, 1 mM EDTA, 300 mM NaCl, and 0.5% Nonidet P-40). Proteins were eluted with 2× loading buffer containing 100 mM DTT by boiling for 5 min. Eluted proteins were run on SDS-PAGE gels, and western blots were probed with an anti-6xHis or anti-GST antibody (Invitrogen).

**GFP Pull-Down Assay**

Agrobacterium tumefaciens strains carrying pK7FWG2-WRKY18 (WRKY18-GFP) and pMDC43-EV (GFP) were co-infiltrated into Nicotiana benthamiana leaves. After 48 h, leaves were harvested, frozen in liquid nitrogen, and homogenized with protein extraction buffer. Homogenates were centrifuged (20,000 rpm, 4°C) for 30 min at 4°C twice and then filtered through a 0.2-μm filter. GFP or GFP-tagged proteins were pulled down with GFP-Trap Magnetic Agarose beads (ChromoTek) or anti-GST antibody (Invitrogen). Beads with attached proteins were washed four times with Tris-EDTA buffer twice (Saleh et al., 2008). After washing, the immunoprecipitated chromatin complex was incubated at 4°C overnight. The immunoprecipitated proteins were washed four times and eluted in 2× Laemmlli sample buffer supplemented with 0.1 mg mL⁻¹ bovine serum albumin and 1 μg of 6xHis-MBP-NPR1 with or without 200 μM SA. After 16 h, the beads were washed with washing buffer four times and eluted in 2× Laemmlli sample buffer supplemented with 100 mM DTT, then boiled in a water bath at 95°C. Western blots were probed with an anti-GFP (Clontech) or anti-6xHis (Invitrogen) antibody.

**Co-IP Assay**

A. tumefaciens strains carrying pCB302-NPR1 (NPR1-GFP), pK7FWG2-WRKY6/WRKY18/TGA5/TGA7 (WRKY6/WRKY18/TGA5/TGA7-GFP), or pMDC43-EV (GFP) were co-infiltrated into N. benthamiana leaves with A. tumefaciens strain carrying pEarlyGate 201-CDK8 (HA-CDK8) and P19 silencer. After 48 h, leaves were harvested, frozen in liquid nitrogen, and homogenized with protein extraction buffer. Homogenates were centrifuged (20,000g, 4°C) for 30 min at 4°C twice and then filtered through a 0.2-μm filter. GFP or GFP-tagged proteins were immunoprecipitated with GFP-Trap Magnetic Agarose beads (ChromoTek) at 4°C for 2 h. The immunoprecipitated proteins were washed four times and eluted in 2× Laemmlli sample buffer supplemented with 100 mM DTT, then boiled in a water bath at 95°C. Western blots were probed with an anti-GFP (Clontech) or anti-6xHis (Invitrogen) antibody. Fifty micrograms of protein extracts was used as an input control.

**ChIP-qPCR Assay**

Five-week-old plants or 14-d-old seedlings of GFP, NPR1-GFP, npr1-2, and cdk8-4 mutant plants infected with virulent Psm. DNA was recovered and analyzed by qPCR with gene-specific primers by Srb10 cyclin-dependent kinase. Genes were used as input control. Western blots were probed with an ant-GST antibody (Invitrogen).

**Statistical Analysis**

Statistical analysis was carried out by Excel 2010 with two-tailed Student’s t test or PASW statistics 18 software with Duncan’s multiple range test.

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**LITERATURE CITED**


**Supplemental Data**

The following supplemental materials are available.

**Supplemental Figure S1.** NPR1 antibody recognizes both npr1-2 and npr1-1 proteins expressed in yeast.

**Supplemental Figure S2.** GFP and NPR1 protein levels in GFP npr1-2 and NPR1-GFP npr1-2 plants.

**Supplemental Figure S3.** PRI gene expression levels in the local leaves of Col-0 wild-type, npr1-2, and cdk8-4 mutant plants infected with virulent Psm.

**Supplemental Figure S4.** CDK8 is required for NPR1 gene expression and tolerance to SA.

**Supplemental Figure S5.** CDK8 regulates NPR1 protein accumulation and PRI gene expression in a kinase-independent manner.

**Supplemental Table S1.** Primers used in this study.
NPR1 Recruits CDK8, Promoting Its Own Expression


Kim HS, Delaney TP (2002) Over-expression of TGA5, which encodes a basic domain/leucine zipper transcription factor TGA1. Plant Cell 15: 2181–2191


resistance against the necrotrophic fungal pathogen *Sclerotinia sclerotiorum*. Plant Physiol. 169: 856–872


