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Two interacting transcriptional coactivators cooperatively control plant immune responses

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The phytohormone salicylic acid (SA) plays a pivotal role in plant defense against biotrophic and hemibiotrophic pathogens. NPR1 and EDS1 function as two central hubs in plant local and systemic immunity. However, it is unclear how NPR1 orchestrates gene regulation and whether EDS1 directly participates in transcriptional reprogramming. Here, we show that NPR1 and EDS1 synergistically activate *pathogenesis-related* (*PR*) genes and plant defenses by forming a protein complex and recruiting Mediator. We discover that EDS1 functions as an autonomous transcriptional coactivator with intrinsic transactivation domains and physically interacts with the CDK8 subunit of Mediator. Upon SA induction, EDS1 is directly recruited by NPR1 onto the *PR1* promoter via physical NPR1-EDS1 interactions, thereby potentiating *PR1* activation. We further demonstrate that EDS1 stabilizes NPR1 protein and NPR1 transcriptionally up-regulates *EDS1*. Our results reveal an elegant interplay of key coactivators with Mediator and elucidate important molecular mechanisms for activating transcription during immune responses.

INTRODUCTION

Plant-pathogen interactions have enabled plants to evolve a sophisticated and multifaceted immune system for defending against pathogen attacks (1). Recognition of conserved pathogen-associated molecular patterns (PAMPs) by extracellular pattern recognition receptors in plants stimulates PAMP-triggered immunity (PTI). However, successful pathogens deploy a suite of virulence effectors to attenuate or dampen PTI, resulting in effector-triggered susceptibility. During host-pathogen coevolution, plants have developed resistance (R) proteins to specifically recognize pathogen-delivered effectors through direct interaction or indirect recognition by detecting the activities of pathogen effectors (2, 3), thus inducing a robust defense, termed effector-triggered immunity (ETI). Most R proteins belong to a large family of intracellular immune receptors known as nucleotide-binding, leucine-rich repeat receptor (NLR) proteins with a variable N-terminal *Drosophila* Toll, mammalian interleukin-1 receptor (TIR) or coiled-coil (CC) domain (4). Activation of PTI or ETI results in the generation of mobile signals that are transported from local infected tissue to distal uninfected parts, inducing systemic acquired resistance (SAR), which is a long-lasting and broad-spectrum resistance against related or unrelated pathogens (5).

The plant defense hormone salicylic acid (SA), as a small phenolic compound, plays a pivotal role in plant defense against biotrophic pathogens such as the oomycete pathogen *Hyaloperonospora arabidopsidis* and hemibiotrophic pathogens such as the bacterial pathogen *Pseudomonas syringae* (6). Pathogen-induced SA accumulates not only in infected local leaves but also in uninfected systemic tissues. Exogenous application of SA or its active analogs is sufficient to activate plant defense responses by inducing massive transcriptional

reprogramming to relocate energy for defense instead of growth. Consequently, SA is an essential signaling molecule for the activation of local defense and SAR (7).

Nonexpresser of pathogenesis-related (PR) genes 1 (NPR1) was identified through genetic screens for *Arabidopsis* mutants that cannot activate the expression of PR genes (8, 9), which encode proteins with antimicrobial activities (6). Similar to NPR3 and NPR4, NPR1 binds SA and functions as an SA receptor (10–12). Before pathogen infection, NPR1 is sequestered in the cytosol as oligomers, which are crucial for protein homeostasis (13). Upon pathogen challenge, oligomeric NPR1 is reduced into active monomers by SA-induced redox changes, and NPR1 monomers enter the nucleus (14). As a transcriptional coactivator, NPR1 interacts with TGA (TGACG-binding motif) and TCP (teosinte branched 1/cycloidea/PCF) transcription factors (TFs) and facilitates the expression of PR genes (15–17). In addition to PR genes, NPR1 also controls the expression of the vast majority of other SA-responsive genes (18, 19). Therefore, it is believed that NPR1 functions as a master regulator of SA signaling.

Enhanced disease susceptibility 1 (EDS1) has been shown to be indispensable for TIR-NLR (TNL) protein-dependent ETI, plant basal defense, and SAR (4, 20–23). In addition to its association with numerous R proteins (24, 25), EDS1 physically interacts with phytoalexin deficient 4 (PAD4) or senescence associated gene 101 (SAG101) (26). Distinct EDS1-PAD4 and EDS1-SAG101 complexes are essential for different R protein-mediated ETI (27). EDS1 and its partners have been shown to affect the expression of numerous pathogen-responsive genes (28, 29), but it remains unclear how EDS1 promotes downstream transcriptional reprogramming to trigger a series of immune responses.

In this study, we show that NPR1 and EDS1 interact with each other to form a protein complex and synergistically activate plant immunity via SA signaling. We demonstrate that EDS1 has transcriptional activation activity and serves as an acidic transcriptional coactivator, which is directly involved in transcriptional reprogramming by interacting with a component of the Mediator complex, cyclin-dependent kinase 8 (CDK8). Moreover, we find that upon SA induction, NPR1 directly recruits EDS1 to the *PR1* promoter to facilitate the expression of *PR1*. Furthermore, we identify

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a positive feedback loop, in which NPR1 directly up-regulates *EDS1* transcription and EDS1 stabilizes NPR1 protein in plant-pathogen interactions. Our study revealed a unique mechanism, in which two interacting transcriptional coactivators co-opt with Mediator and cooperatively control transcriptional reprogramming to activate plant defense responses.

RESULTS

NPR1 physically interacts with EDS1 to form a protein complex

Both NPR1 and EDS1 function as central hubs in plant immunity (4, 30), and they have also been identified as targets of pathogen effectors (24, 31). In a yeast two-hybrid (Y2H) screen, we have identified EDS1 as an NPR1 interactor. NPR1 specifically interacted with EDS1, but not with PAD4 or SAG101, two other members of the EDS1 family of lipase-like proteins in Y2H assays (Fig. 1A and fig. S1A). The specific NPR1-EDS1 interaction was then confirmed by *in vitro* pull-down assays, where thioredoxin (Trx)-His₆-NPR1 bound glutathione *S*-transferase (GST)-EDS1, but not GST-PAD4 or GST (Fig. 1B). Their interaction in planta was determined using coimmunoprecipitation (co-IP) assays in *Nicotiana benthamiana*, in which EDS1-Myc was coimmunoprecipitated with NPR1-FLAG (Fig. 1C). Using a bimolecular luminescence complementation (BiLC) assay in *N. benthamiana*, the *in planta* interaction between NPR1 and EDS1 was further confirmed (Fig. 1D). Together, these data demonstrate that NPR1 interacts with EDS1 *in vitro* and *in vivo*.

We next carried out a bimolecular fluorescence complementation (BiFC) assay to check the subcellular localization of NPR1-EDS1 complex by transiently expressing these two proteins in *N. benthamiana* using agroinfiltration. Compared with the EDS1-PAD4 complex that was detected mainly in the nucleus and cytoplasm, the observed NPR1 association with EDS1 in the nucleus apparently formed nuclear bodies (Fig. 1E), which most likely act as the sites for accelerating gene activation or repression (32). These data imply that the primary function of the NPR1-EDS1 protein complex is to regulate the expression of plant defense genes. To validate the native NPR1-EDS1 interaction in *Arabidopsis*, we produced transgenic lines expressing the *EDS1* native promoter-driven EDS1-FLAG in Col-0 *eds1-2* mutant (p*EDS1:EDS1-FLAG/eds1-2*) and crossed it with p*NPR1:Myc-NPR1/npr1-3* transgenic lines to obtain the p*NPR1:Myc-NPR1/npr1-3*; p*EDS1:EDS1-FLAG/eds1-2* plants. In reciprocal co-IP experiments, we detected that SA enhances the NPR1-EDS1 association possibly because of the increased protein levels of NPR1 and EDS1 after SA treatment (Fig. 1F). Together, these data suggest that SA induces the accumulation of NPR1-EDS1 protein complex within the nuclear bodies to facilitate the expression of plant defense genes.

We additionally conducted Y2H assays to identify the domain of EDS1 that is necessary for its interaction with NPR1. Several EDS1 fragments including the EP (EDS1- and PAD4-defined) domain, the lipase-like domain, the helical region encompassing amino acid residues from 310 to 350 (310-350) and a CC domain (358-383) (fig. S1B) were tested on the basis of the secondary and crystal structures (23, 26). The results showed that the helical region (310-350) is sufficient and necessary for EDS1 to interact with NPR1. To narrow down the interacting region in the helical structure, we divided it into two α helices (310-330 and 331-350). EDS1 lacking residues 310 to 330 (Δ 310-330) failed to interact with NPR1, while the minimal region (310-330) exhibited obvious interaction. Therefore, the

minimal α helix (310-330) in EDS1 is necessary and sufficient for the interaction with NPR1. On the basis of the crystal structure of EDS1 (26), this minimal α helix (310-330) is located on the surface of the N-terminal domain of EDS1 (fig. S1C), further supporting the critical role of this region for the EDS1-NPR1 interaction.

Conversely, we also generated different truncations of NPR1 and identified several domains of NPR1 that are involved in NPR1-EDS1 interaction (fig. S1, D and E). We found that the BTB/POZ (broad complex, tramtrack, and bric-a-brac/pox virus and zinc finger) domain, the ankyrin (ANK) repeats, and an important C-terminal domain (CTD) interact with EDS1 in Y2H assays. BTB/POZ and ANK motifs are well known as protein-protein interaction motifs of a number of proteins in mammals and plants (8). To further decipher whether the interaction of NPR1 with EDS1 is relevant to NPR1's function, we investigated the interaction of EDS1 with mutant *npr1* proteins encoded by several *npr1* alleles (i.e., *npr1-2*, *nim1-2*, *npr1-1*, and *npr1-5*) that are compromised in SA signaling and SAR induction (9, 33). Consistently, *npr1-2* (C150Y) mutation in BTB domain or other point mutations in ANK region such as *nim1-2* (H300Y), *npr1-1* (H334Y), and *npr1-5* (P342S) completely lost the ability to interact with EDS1 (fig. S1D), indicating that the interaction of EDS1 with NPR1 is important for the function of NPR1. In addition, it has been revealed that the CTD overlapping a repression region of NPR1 (17) is probably involved in SA perception (12). Collectively, these findings suggest that multiple regions of NPR1 are required for the dynamic interaction with EDS1 in plant immune responses.

NPR1 and EDS1 cooperatively activate plant immunity

EDS1 plays an essential role in ETI triggered by TNL proteins (20). EDS1 is required for recognition of the *P. syringae* pv. tomato avirulence effector protein Rps4 (*Pst* AvrRps4) by the nuclear R protein pair resistant to *Ralstonia solanacearum* 1s (RRS1S)-resistant to *P. syringae* (RPS4) in *Arabidopsis* (34). To dissect the roles of the NPR1-EDS1 interaction in controlling plant immunity, genetic interactions were analyzed between recessive *npr1* mutant alleles (*npr1-2* and *npr1-3*) (8) and the null *eds1-2* allele (28) in the *Arabidopsis thaliana* ecotype Col-0 background. In comparison to *eds1-2*, the *npr1-2* and *npr1-3* are moderately susceptible to *Pst* DC3000 *avrRps4*, whereas two transgenic lines overexpressing N-terminal green fluorescent protein (GFP)-tagged NPR1 (35S:*GFP-NPR1* #11 and #36) in Col-0 background were robustly resistant to the avirulent pathogen (Fig. 2A). These suggest that NPR1 prominently contributes to RRS1S/RPS4-mediated ETI. It is well known that NPR1 is crucial to basal resistance and SA/SAR signaling and also implicated in CC NLR (CNL)-mediated ETI and cell death (11). Note that overexpression of *NPR1* has been reported to confer heightened nonspecific resistance (35). Since *NPR1*-overexpressing plants also showed notably enhanced resistance to the virulent pathogen *Pst* DC3000 (fig. S2A), the accelerated TNL ETI by overexpression of *NPR1* could involve the potentiated basal resistance in *NPR1*-overexpressing plants. To further test the function of nuclear NPR1 in ETI, we examined the resistance of the transgenic plants expressing *NPR1-GFP* and its nuclear localization signal (NLS) mutant form *NPR1 (nls)-GFP* to *Pst* DC3000 *avrRps4* in *npr1* background. As shown in fig. S2B, the enhanced ETI conferred by *NPR1-GFP* was completely lost in *NPR1 (nls)-GFP* transgenic plants, revealing that nuclear NPR1 contributes to ETI likely through transcriptional regulation.

In further epistasis analysis, the double mutants (*npr1-2 eds1-2* and *eds1-2 npr1-2*) obtained from two reciprocal crosses (*npr1-2* \times *eds1-2*

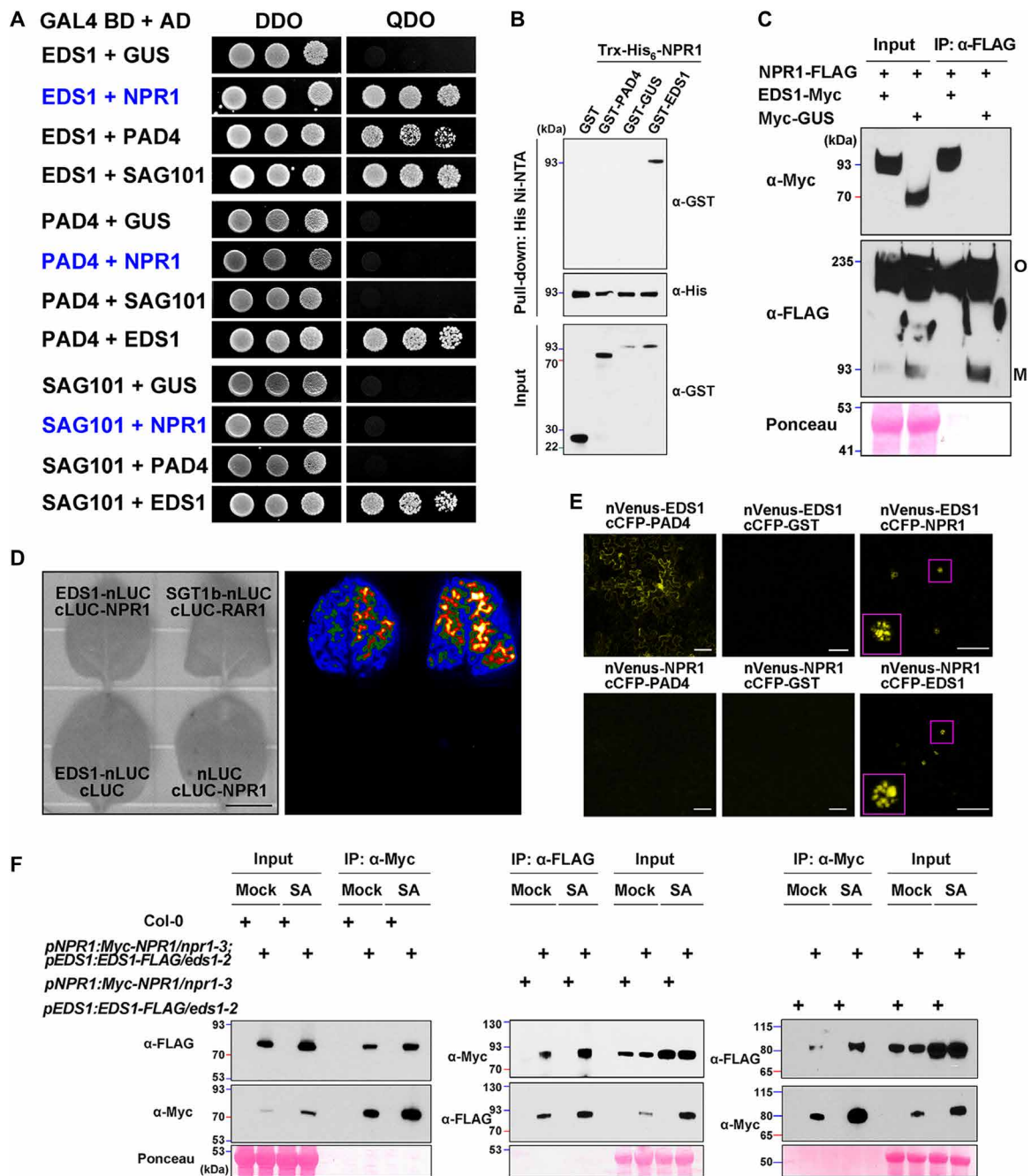


Fig. 1. NPR1 directly interacts with EDS1. (A) NPR1 interacts with EDS1 in Y2H assays. The growth of yeast strains on nonselective double dropout medium (DDO) and selective quadruple dropout medium (QDO) is shown. GAL4 BD, GAL4 DNA binding domain; AD, activation domain. (B) NPR1 interacts with EDS1 in the in vitro pull-down assays. Trx-His₆-NPR1 was used to pull down GST and GST fusion proteins. Trx-His₆-NPR1 and GST fusion proteins were detected by Western blotting with anti-His and anti-GST antibodies, respectively. NTA, nitrilotriacetic acid. (C) NPR1 interacts with EDS1 in *N. benthamiana*. The NPR1-3FLAG under the control of its native promoter was transiently expressed with EDS1-Myc under the control of its native promoter or Myc-GUS under the control of the 35S promoter in *N. benthamiana*. Co-IP assay was performed using anti-FLAG magnetic beads. O, oligomeric NPR1; M, monomeric NPR1. (D) NPR1 interacts with EDS1 in BiLC assays. The indicated vectors were coexpressed in *N. benthamiana* leaves, and luciferase complementation imaging assays were performed. Scale bar, 1 cm. (E) NPR1 interacts with EDS1 in nuclei in BiFC assays. *N. benthamiana* was cotransformed with indicated constructs. Magnified nuclear body is shown in pink boxes. Scale bars, 150 μm. (F) NPR1 interacts with EDS1 in *Arabidopsis*. The 2-week-old *Arabidopsis* seedlings were treated with 0.5 mM SA or water (Mock) for 9 hours, and total protein extract was subject to co-IP assays using Myc-Trap_MA or anti-FLAG magnetic beads. Ponceau S staining of ribulose-1,5-bisphosphate carboxylase-oxygenase is used for confirmation of equal loading. Protein sizes marked on the left are in kilodalton.

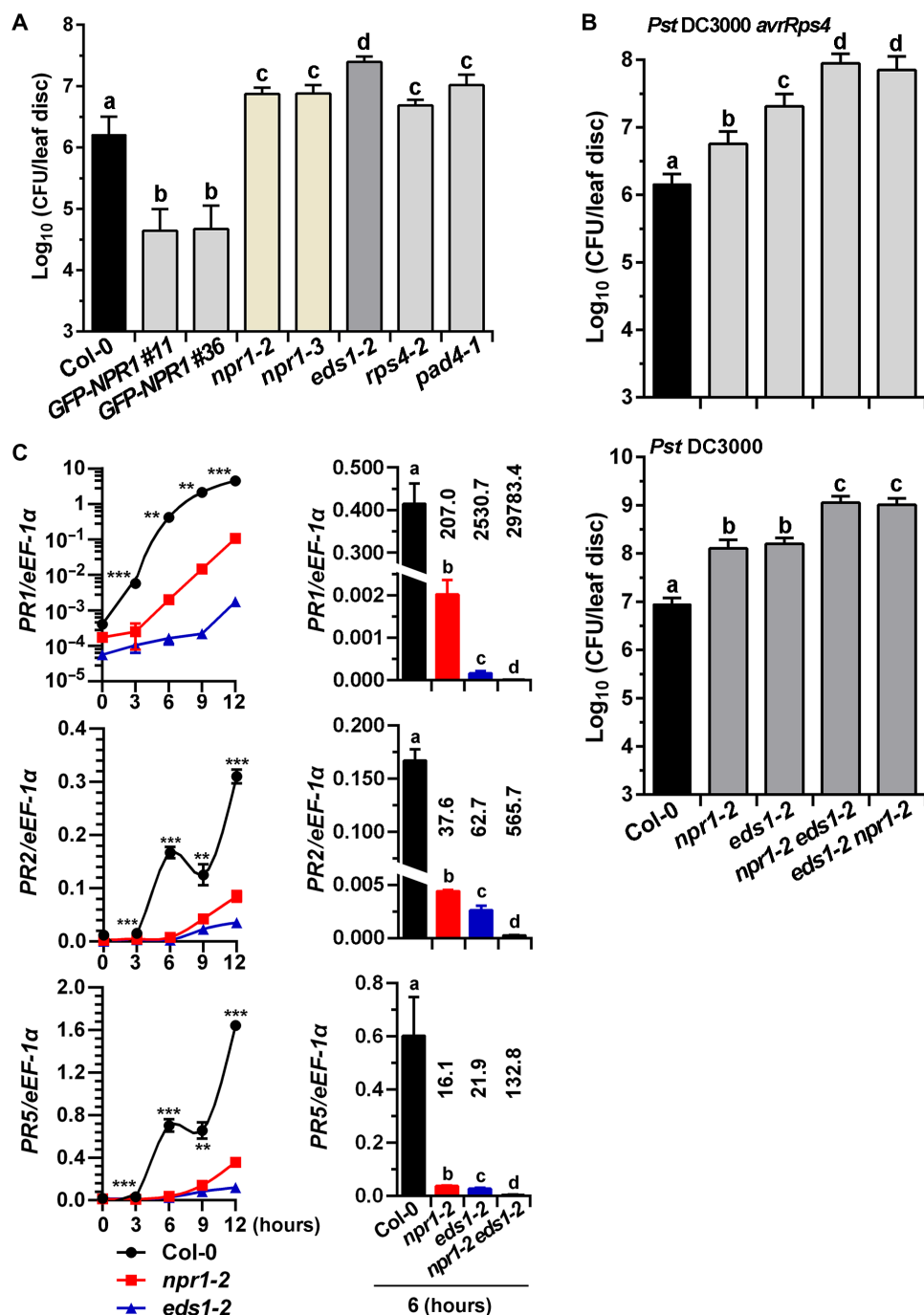


Fig. 2. Genetic and molecular interactions of NPR1 with EDS1. (A) NPR1 contributes to ETI. Growth of *Pst DC3000 avrRps4* on Col-0, different mutants, and transgenic *Arabidopsis* overexpressing GFP-NPR1 under the control of the *CaMV 35S* promoter. (B) NPR1 and EDS1 additively activate immune responses. Leaves from soil-grown *Arabidopsis* (A and B) were hand-infiltrated with indicated bacterial suspensions [optical density at 600 nm (OD₆₀₀) = 0.0005], and bacterial titers were measured at 2 days post-inoculation (dpi). CFU, colony-forming units. (C) NPR1 and EDS1 synergistically activate PR genes. Leaves from 4-week-old plants inoculated with *Pst DC3000 avrRps4* (OD₆₀₀ = 0.01) were collected at indicated time points, and PR gene expression was checked using real-time quantitative polymerase chain reaction (qPCR). Expression of PR1 was plotted on a log₁₀ scale; gene expression levels were normalized against the constitutively expressed *eEF-1α*. Right: The expression of PR genes at 6 hours after pathogen infection. Notably, the numbers above the error bars indicate the fold change of gene expression compared with Col-0. Error bars represent SDs; *n* = 6 biologically independent samples from six different plants (A and B); *n* = 4 biologically independent replicates (C). Statistically significant differences indicated by different lowercase letters are analyzed by one-way analysis of variance (ANOVA; *P* < 0.05) or shown between Col-0 and single mutant (*npr1-2* or *eds1-2*) plants (multiple *t* test, ***P* < 0.01 and ****P* < 0.001).

and *eds1-2* × *npr1-2*) were more susceptible to *Pst* DC3000 *avrRps4* and *Pst* DC3000 than either *npr1-2* or *eds1-2* single mutants (Fig. 2B), demonstrating that NPR1 and EDS1 additively contribute to ETI and basal resistance. To determine whether EDS1 is involved in NPR1-mediated defense pathways, *NPR1-GFP/npr1-2* transgenic plants were crossed with *eds1-2* mutants, and homozygous *NPR1-GFP/npr1-2;eds1-2* plants were identified and analyzed. *NPR1-GFP/npr1-2;eds1-2* plants exhibit a susceptibility somewhat less than that conferred by *eds1-2* (fig. S2C), suggesting that EDS1 functions both dependently and independently of NPR1 to regulate ETI. To further confirm the function of NPR1-EDS1 interaction in plant defense, we examined the susceptibility of these mutants to another avirulent pathogen *P. syringae* pv. *maculicola* (*Psm*) ES4326 *avrRpt2*, which activates ETI mediated by the CNL protein RPS2 (36). We found that the growth of *Psm* ES4326 *avrRpt2* in *eds1-2* is not significantly higher than wild-type plants. However, the pathogen growth in *npr1-2 eds1-2* or *eds1-2 npr1-2* was higher than either *npr1-2* or *eds1-2* (fig. S2D), indicating the collaborative contributions of NPR1 and EDS1 in RPS2-mediated ETI. Thus, these genetic interaction data are consistent with the hypothesis that NPR1 and EDS1 function as partners in diverse immune responses.

In addition to genetic interactions, the molecular function of the NPR1-EDS1 interaction was investigated. We used real-time quantitative polymerase chain reaction (qPCR) to monitor the time course expression of *PR* genes (*PR1*, *PR2*, and *PR5*), a subset of EDS1-induced *WRKY* genes and two EDS1-repressed genes (*DND1* and *ERECTA*) in ETI (28, 29). These *PR* genes and EDS1 target genes were misregulated in *npr1-2* and *eds1-2* in similar manners (Fig. 2C and fig. S2, E and F). Loss of *EDS1* function has a stronger effect than loss of *NPR1* function on expression of *PR* genes after pathogen infection (Fig. 2C), consistent with the bacterial growth data (Fig. 2A and fig. S2C). The reduction in the expression of *PR* genes in the *npr1-2 eds1-2* was more pronounced than single mutants (Fig. 2C, right). These results indicate that the synergistic regulation of defense gene expression by NPR1 and EDS1 is essential for immune responses.

NPR1 and EDS1 synergistically promote SA signaling in plant defenses

EDS1 was shown to function upstream of SA in plant immunity because it contributes to pathogen-induced SA accumulation (37). *PR* proteins have been considered as hallmarks of SA signaling (9, 38, 39). Notably, the *eds1-2* mutation inhibited *Pst* DC3000 *avrRps4*-induced *PR1/2/5* gene expression (Fig. 2C); thus, EDS1 may also function in SA signaling transduction. To study potential roles of EDS1 in response to SA, the expression of SA hallmark genes (i.e., *PR1/2/5*) in two *eds1* mutant alleles treated with SA was examined. The result from qPCR analysis showed that the expression of SA-induced *PR* genes was significantly decreased in the *eds1-2* rosette leaves from soil-grown plants (fig. S3A) and seedlings (fig. S3B). SA-induced *PR1* protein accumulation in *Ws-0 eds1-1* or *Col-0 eds1-2* was significantly attenuated and delayed (Fig. 3A), compared to the corresponding wild-type plants. Concomitantly, the gradually increased EDS1 protein over time was highly elevated by SA (Fig. 3A). Hence, the SA-induced EDS1 plays a positive role in activating SA signaling to regulate defense genes. We also examined SA-induced pathogen resistance in *eds1* mutants. In contrast to the *npr1-2* plants, exogenous application of SA significantly rendered *eds1-1* and *eds1-2* plants resistant to *Pst* DC3000 (Fig. 3B), consistent with the idea

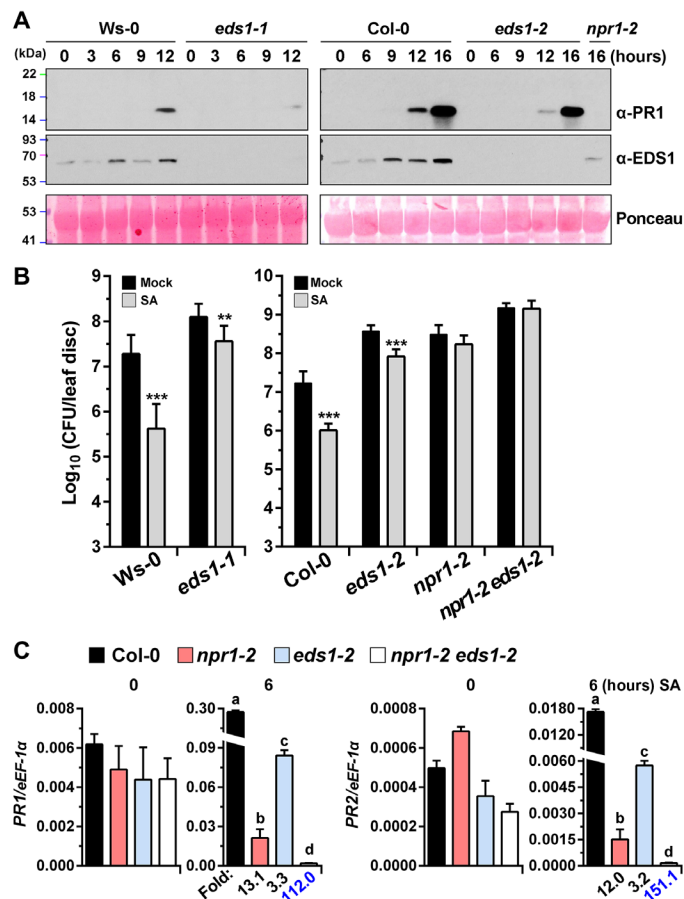


Fig. 3. NPR1 and EDS1 synergistically activate downstream SA signaling.

(A) EDS1 is a positive regulator of SA signaling. Total protein was prepared from leaf tissues of 4-week-old plants infiltrated with 0.3 mM SA and subjected to immunoblotting with indicated antibodies. This result is representative of at least two independent experiments. (B) EDS1 contributes to SA-induced pathogen resistance. Plants were treated with soil drenches plus foliar sprays of 0.5 mM SA or water (Mock). After 24 hours, leaves were inoculated with *Pst* DC3000 (OD₆₀₀ = 0.0005), and the in planta bacterial titers were determined at 3 dpi. Error bars represent SEs; *n* = 2 biologically independent experiments carried out on separated days. Statistical differences from Mock in each genotype are shown (Student's *t* test, ***P* < 0.01 and ****P* < 0.001). (C) NPR1 and EDS1 synergistically activate *PR1* and *PR2*. Two-week-old seedlings grown on 1/2 MS media were exogenously treated with hydroponic 0.5 mM SA solution for 6 hours. Total RNA was extracted and subjected to quantitative reverse transcription PCR (qRT-PCR). Error bars indicate SDs; *n* = 4 biologically independent replicates. Different letters indicate statistical differences (ANOVA, *P* < 0.01). Fold numbers on the x axis refer to fold reduction of gene expression in relevant mutants compared with the value obtained in *Col-0*.

that EDS1 functions upstream of SA. However, the SA-induced pathogen resistance in *eds1-1* or *eds1-2* was not as strong as that in wild-type plants (Fig. 3B). These findings are in agreement with our conclusion that EDS1 can function as a positive regulator of SA signaling in plant immune responses.

To investigate the functions of the NPR1-EDS1 interaction in SA signaling, we examined the expression of other SA-responsive and NPR1 target genes in *eds1-2* mutant. In addition to NPR1-dependent *PR* genes (Fig. 3A and fig. S3, A and B), some *WRKY* genes, as well as several genes involved in pathogen-induced SA accumulation,

were significantly reduced in *eds1-2* compared with Col-0 after SA treatment (fig. S3C), suggesting that EDS1 and NPR1 up-regulate the expression of a common set of SA-induced genes. To determine the contribution of EDS1 to the *PR* gene expression in the absence of NPR1, the expression of both *PR1* and *PR2* was examined in *npr1-2 eds1-2* in response to SA. The expression of *PR1* and *PR2* were markedly reduced in *npr1-2 eds1-2* seedlings (Fig. 3C), compared to *npr1-2* or *eds1-2* seedlings. Notably, the fold change for the reduction of *PR1* expression in *npr1-2 eds1-2* (112.0-fold) was much greater than the product of the fold change in *npr1-2* (13.1-fold) and *eds1-2* (3.3-fold). Similar results were found with seedlings that were grown on media containing low concentrations of SA for a long-term treatment (fig. S3D). Therefore, these results firmly demonstrate that NPR1-EDS1 interaction synergistically activates SA signaling and pathogen resistances.

The NPR1-EDS1 complex associates with specific chromatin regions upon SA induction

To further explore the effects of NPR1-EDS1 complex on the expression of defense genes, we conducted a series of chromatin immunoprecipitation (ChIP) assays. Multiple NPR1-interacting TGA factors (33, 40) and numerous WRKY TFs that bind specifically to the W-box motif (TTGACC/T) have been shown to play essential roles in plant defense pathways (41–43). On the basis of these studies, we chose a set of promoter fragments that contain the common TGA motif (TGACG), the preferred TGA2-binding motif (TGACTT) (10), or the W-box in our ChIP assays. As shown in Fig. 4A, Myc-NPR1 specifically associates with chromatin fragments at the *PR1* promoter in *pNPR1:Myc-NPR1/npr1-3* transgenic plants after SA treatment. In contrast, Myc-NPR1 did not significantly associate with the *PR1* promoter in the absence of SA, probably owing to constitutive protein degradation (44) and the persistent existence of cytosolic oligomers under noninducing conditions (13). Unexpectedly, EDS1-FLAG in *pEDS1:EDS1-FLAG/eds1-2* transgenic lines and Myc-NPR1 bind almost identical sites on the *PR1* promoter (Fig. 4A). Moreover, NPR1 and EDS1 associated with the promoters of *PR2* and *PBS3* with similar enrichment profiles especially after SA treatment (fig. S4, A and B). Besides, both EDS1-FLAG and NPR1-Myc could associate with *PR1* promoter upon short-term SA induction, albeit with lower enrichment (Fig. 4B). Together, these results demonstrate that EDS1 is a chromatin-associated protein and the NPR1-EDS1 complex associates with specific chromatin regions upon SA induction.

Given that the chromatin binding of EDS1 was strongly enhanced by SA (Fig. 4, A and B), we next tested whether SA contributes to nuclear translocation of EDS1 using *35S:EDS1-eGFP/eds1-2* transgenic plants, which constitutively express EDS1 fused with enhanced GFP (eGFP) in the *eds1-2* background. After SA treatment, we found that neither the accumulation of constitutively expressed EDS1-eGFP protein (fig. S4C) nor its nuclear import (fig. S4D) was apparently induced by SA, in agreement with the finding from another parallel experiment using *35S:GFP-EDS1/eds1-2* plants (fig. S4E). Thus, SA does not facilitate nuclear translocation of EDS1. It is worthwhile to mention that endogenous EDS1 protein in nuclei is obviously induced by SA (fig. S4F), which is attributed to the fact that the total EDS1 protein expression is enhanced by SA (Figs. 1F and 3A). These data indicate that the accumulation of nuclear EDS1 is required for SA-triggered chromatin binding by EDS1.

EDS1 functions as a transcriptional coactivator with acidic activation domains

In view of the autoactivation of EDS1 observed in our initial Y2H system (fig. S4G), we speculated that EDS1 has transcriptional activator activity. To further test this, we detected transcriptional activation activity using a yeast monohybrid assay, in which GAL4 DNA binding domain fusion proteins were transformed into a yeast strain carrying GAL4 promoter-dependent reporter genes (45). On the basis of *HIS3*, *MEL1*, and *lacZ* reporter assays (Fig. 4B), we found that full-length EDS1 and TGA3 have transcriptional activation activities in contrast to the GAL4 DBD empty vector, PAD4, and TGA2 (Fig. 4C, top). A more in-depth N-terminal and C-terminal deletion analysis identified two transactivation domains (TADs) located at the α -helical region (331-350) and the C-terminal region (542-593) that are either necessary or sufficient for the transactivation activity of EDS1, respectively (Fig. 4B). Acidic activation domains (AADs), also known as “acidic blobs,” play essential roles in the functions of important transcriptional activators such as p53, GCN4 (general control nondepressible 4), GAL4, and VP16 (virion protein 16) (46–48). The acidic amino acids and surrounding hydrophobic residues within AAD have been shown to be critical structural elements for AAD, and they are presumably involved in both ionic and hydrophobic interactions with AAD’s target molecules (47). We found that acidic and hydrophobic amino acids are enriched in the N- and C-terminal TADs of EDS1 (Fig. 4C, bottom), indicating that EDS1 is a transcriptional activator with AADs. Together, EDS1 harbors two AADs and has transcriptional activator function.

To further analyze the activator function of EDS1 in planta, we investigated the transcriptional regulation of defense genes by EDS1 using *35S:EDS1-eGFP/eds1-2* transgenic plants. In the transactivation experiments, EDS1-eGFP alone had no effect on gene transcription without SA treatment, but it significantly induced the expression of *PR1* (Fig. 4D) and other defense genes (i.e., *PR2*, *PR5*, *PAD4*, and *PBS3*) in the presence of SA (fig. S4H). In addition, EDS1 likely binds to defense gene promoters through intermediate transcriptional regulators owing to lack of a DNA binding domain (23). Together with the above ChIP and yeast results, these data demonstrate that EDS1 can bind chromatin and acts as a transcriptional coactivator to activate defense genes upon SA induction.

EDS1 strongly interacts with Mediator

Mediator complex has emerged as a key transcriptional regulator linking different transcription activators and RNA polymerase II (RNAPII) preinitiation complex (49). CDK8 is a key component in the kinase module (CDK8 module) of the Mediator complex. Increasing studies have demonstrated that CDK8 can play positive roles in gene activation in mammalian and plant cells (50, 51). Notably, our previous research has shown that CDK8 associates with NPR1 in plants and several components of the CDK8 module positively regulate SA signaling in SAR (51). To investigate the mechanism for EDS1-mediated transactivation, we examined the possible association of CDK8 with EDS1 by co-IP assays. We observed that EDS1 associates with CDK8 in plants (Fig. 4E). To test whether EDS1 physically interacts with CDK8, a reciprocal Y2H assay was used and the strong direct EDS1-CDK8 interaction was confirmed (Fig. 4F). Moreover, the EDS1 (Δ 331-350) deletion mutant is unable to interact with CDK8 (Fig. 4F), suggesting that this TAD of EDS1 confers to the direct interaction with Mediator. These results further support that EDS1 functions as a transcriptional coactivator, which

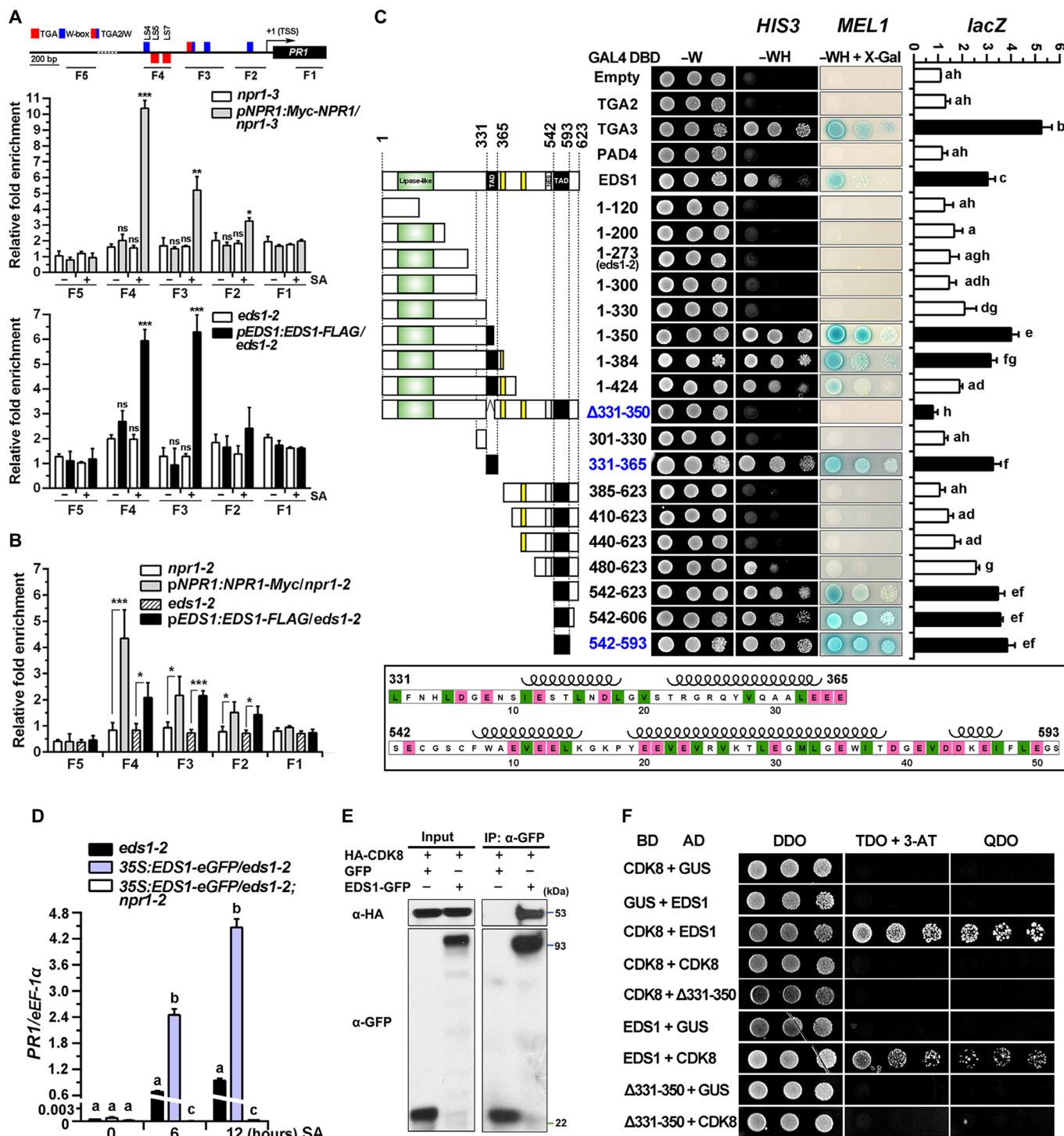


Fig. 4. EDS1 functions as an acidic transcriptional coactivator. (A and B) ChIP-qPCR analysis was performed with homozygous lines, which were harvested after 0.5 mM SA (+) or water (–) treatment for 9 (A) or 3 hours (B). bp, base pairs. Top: Schematic representation of the cis-elements and chromatin fragments of amplicon in the *PR1* genomic region. LS4, equivalent to W-box (blue); LS5/7, TGA motif (red); TGA2/W (chimeric color), TGA2 binding site overlapping W-box; TSS, transcriptional start site. The inverted consensus sequences are shown below. (C) Left: Schematic illustration of EDS1 and mutants. Lipase-like domain (green); putative NLS (yellow); NES, nuclear export signal (white); TAD, transactivation domain (solid). Right: Qualitative and quantitative assays of transactivation activity. Bottom: Acidic (red) and hydrophobic (green) amino acid residues in TADs. Coils refer to α helices. (D) SA-induced EDS1 activates *PR1*. Four-week-old plants were infiltrated with 0.3 mM SA. (E and F) EDS1 TAD interacts with CDK8. Indicated vectors under the control of 35S promoter were coexpressed in *N. benthamiana*. Direct interaction was determined by Y2H. Error bars indicate SDs; $n = 3$ biologically independent replicates (A, B, and D) or experiments with different yeast clones (C). Significances of differences (t test, $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$) from mutants treated with water or SA (A and B), and statistical comparisons made separately among genotypes for each time point (D) are shown. Different letters indicate significant differences ($P < 0.05$). ns, not significant.

is mediated by interacting with Mediator in regulating RNAPII for pathway-specific transcription.

EDS1 is directly recruited by NPR1 onto the *PR1* promoter via a physical NPR1-EDS1 interaction

As shown above, EDS1 and NPR1 occupy the same chromatin loci and synergistically activate plant defense genes (Figs. 2C, 3C, and 4A), supporting that EDS1 is a functional NPR1 cofactor in SA-mediated gene regulation. To further explore the effects of SA and NPR1-EDS1 complex on the enrichment of NPR1 and EDS1 at the *PR1* promoter, we performed a series of ChIP and cell fractionation experiments using different transgenic plants constitutively expressing EDS1-eGFP or NPR1-GFP in diverse genetic backgrounds.

First, we determined whether the NPR1-EDS1 interaction affected the recruitment of NPR1 and/or EDS1 to the *PR1* promoter. In the ChIP experiments using *35S:EDS1-eGFP/eds1-2;npr1-2* and *35S:EDS1-eGFP/eds1-2* transgenic lines, *npr1-2* greatly suppressed the occupancy of EDS1-eGFP at specific sites on the *PR1* promoter (Fig. 5A), suggesting that NPR1 is essential for the association of EDS1 with the *PR1* promoter after SA treatment. In contrast, EDS1 appears to only slightly affect NPR1-GFP residence on the *PR1* promoter based on assays using *35S:NPR1-GFP/npr1-2* and *35S:NPR1-GFP/npr1-2;eds1-2* lines (fig. S5A). These findings indicate that NPR1 is indispensable for EDS1 recruitment at the *PR1* promoter upon SA induction but not vice versa.

Next, we sought to examine the effects of SA and NPR1 on recruiting EDS1 to the *PR1* promoter. Similar to the results obtained with *pEDS1:EDS1-FLAG/eds1-2* lines (Fig. 4A), the EDS1-eGFP enrichment at the *PR1* promoter in *35S:EDS1-eGFP/eds1-2* line was also dependent on SA (Fig. 5A). Since SA did not increase the levels of EDS1-eGFP (fig. S4C) or its nuclear translocation (fig. S4, D and E), we believe that regulation of nuclear EDS1 by SA is critical for its association with the *PR1* promoter. Notably, our cell fractionation assays demonstrated that neither the EDS1-eGFP expression (fig. S5B) nor its nuclear translocation was promoted by NPR1 after SA treatment (fig. S5C), significantly ruling out the possibility that NPR1 facilitates the nuclear movement of EDS1 upon SA induction. Note that the association of NPR1 with chromatin on the *PR1* promoter was dependent on SA (Fig. 4A). Together, these results indicate that SA-induced association of NPR1 with chromatin is crucial for the SA-triggered EDS1 recruitment onto *PR1* promoter, which is independent of the nucleocytoplasmic trafficking of EDS1.

We next focused on investigating the mechanisms used by NPR1 in recruiting EDS1 onto the *PR1* promoter. The TGA2 subclade TFs, the major regulators of NPR1-mediated SAR and expression of *PR* genes (40), are implicated in the recruitment of NPR1 onto the *PR1* promoter (15, 52). NPR1 and EDS1 were enriched at the chromatin site F4 region containing activation sequence-1 (*as-1*)-like cis-elements on the *PR1* promoter (Fig. 4A), an important region for basal and SA-induced *PR1* expression (53). The SA-responsive *as-1* region is proposed to be occupied by constitutive trans-acting factors such as the TGA2/5 and additional factors in uninduced states (52–54). Since the constitutively expressed EDS1-eGFP in the nucleus (figs. S4, D and E, and S5C) did not reside at the *PR1* promoter under uninduced states (Fig. 5A), this deduces that EDS1 might not be recruited by the aforementioned trans-acting factors. *npr1-2* mutation completely abolished an association of EDS1-eGFP with the F4 (*as-1*) region upon SA induction (Fig. 5A), and EDS1 was not shown to physically interact with TGA2/5 by Y2H assays (fig. S5D), further

emphasizing that the direct recruitment of EDS1 onto the *PR1* promoter is predominantly dependent on the physical NPR1-EDS1 interaction.

On the basis of the above results, we conclude that NPR1 directly recruits EDS1 to the *PR1* promoter, which is crucial for SA-induced EDS1 chromatin binding and *PR1* activation. Consistently, the enhanced activation of *PR1* and other defense-related genes in the *35S:EDS1-eGFP/eds1-2* plants upon SA induction is significantly compromised by the *npr1-2* mutation (Fig. 4D and fig. S4H), suggesting that EDS1 cooperates with NPR1 for potentiation of *PR1* activation. This proposition is in line with the immunoblot results showing that NPR1 is required for the accumulation of PR1 protein induced by EDS1-eGFP (Fig. 5B). Overall, EDS1 is directly recruited by NPR1 and participates in transcriptional reprogramming with Mediator complex and therefore reinforces SA-mediated defense responses.

As shown above, the α -helical (310–330) and TAD (331–350) regions of EDS1 play key roles for the interaction with NPR1 and Mediator, respectively (fig. S1, B and C, and Fig. 4, C to F). In addition, these regions are not conserved in PAD4 or SAG101 by multiple protein sequence alignments (fig. S4G, bottom). To further explore the *in vivo* function of these specific regions of EDS1 in transcriptional reprogramming and plant defenses, we tested the complementation of two deletion variants EDS1 (Δ 310–330) and EDS1 (Δ 331–350) in *eds1* mutants. Compared with transgenic plants expressing full-length *EDS1-eGFP* in Col-0 *eds1-2* background, the activation of SA-dependent *PR* defense genes and other defense-related genes was significantly compromised in transgenic plants expressing *EDS1* (Δ 310–330)-*eGFP* or *EDS1* (Δ 331–350)-*eGFP* (Fig. 5C). Similarly, the induction of PR1 protein by full-length *EDS1-eGFP* was robustly affected by the two deletion variants in *Ws-0 eds1-1* mutant (fig. S5E). In further TNL (RRS1S-RPS4) and basal resistance complementation assays, neither *EDS1* (Δ 310–330)-*eGFP* nor *EDS1* (Δ 331–350)-*eGFP* transgenic plants were as resistant as intact *EDS1-eGFP* transformants (Fig. 5D), suggesting that these two specific regions of EDS1 are necessary for TNL-mediated ETI and basal defense. Therefore, the EDS1-NPR1 and EDS1-Mediator heterodimers are essential for reprogramming gene regulation and diverse immune responses.

NPR1 transcriptionally up-regulates *EDS1*

Because EDS1 is strongly induced by SA (Figs. 1F and 3A), we speculate that NPR1 regulates *EDS1* expression. To test this hypothesis, we examined the dynamic expression of EDS1 protein in *npr1-2* and *npr1-3* mutants at different time points. As anticipated, SA-induced EDS1 protein level was obviously reduced in *npr1-2* (Fig. 6A) or *npr1-3* mutants (fig. S6A). SA-up-regulated *EDS1* transcript level was also significantly diminished in *npr1-2* (Fig. 6B) or *npr1-3* mutants (fig. S6B), compared with the wild-type control. Therefore, NPR1 preferentially up-regulates *EDS1* transcription. Additional ChIP assays demonstrated the SA-dependent association of NPR1-GFP with the *EDS1* promoter at TGA motifs (Fig. 6C, top). Thus, NPR1 directly activates *EDS1* transcription upon SA induction. Since TGA2-NPR1 complex is crucial for *PR1* activation (17, 40), we further test whether the TGA2 also directly targets the *EDS1* promoter. ChIP experiments showed that TGA2-GFP strongly associated with two TGA motifs within the *EDS1* promoter (Fig. 6C, bottom). These data suggest that TGA2-NPR1 complex directly activates *EDS1*.

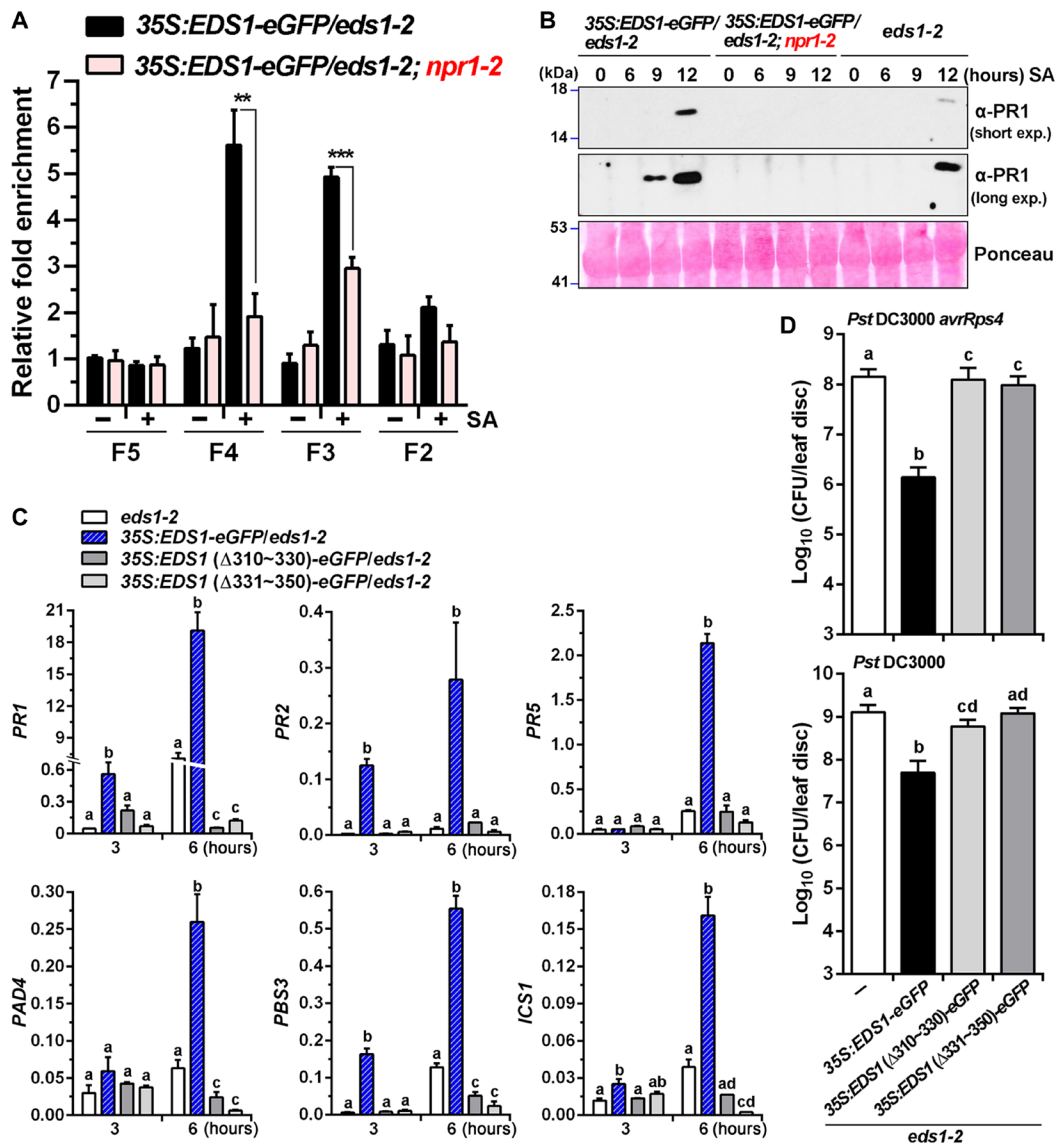


Fig. 5. NPR1-EDS1 and EDS1-CDK8 heterodimers contribute to potentiation of defense responses. (A) NPR1 directly recruits EDS1 onto *PR1* genomic loci using indicated soil-grown transgenic plants treated with foliar sprays plus soil drenches of 0.5 mM SA (+) or water (-) for 9 hours. The experiment was performed as in Fig. 4A. (B) EDS1 potentiates SA/NPR1-mediated PR1 accumulation. Leaves from soil-grown plants were infiltrated with 0.3 mM SA solution. Short- and long-exposure (exp.) images of same immunoblotting blot are shown. (C) Expression of *EDS1*-activated genes in response to SA in complementation analysis. Gene expression was analyzed by qRT-PCR and normalized to *eEF-1α*. Leaves of 4-week-old plants were infiltrated with 0.6 mM SA. Bars represent SEs; *n* = 3 biologically independent experiments performed on separate days. Dozens of individual T₁ transgenic lines that expressed the GFP-tagged protein for each genotype were confirmed by immunoblotting using α-GFP antibodies. Different letters indicate significant differences (two-way ANOVA, *P* < 0.05). The statistical comparisons were made separately among genotypes for each time point. (D) TNL and basal resistance phenotypes in complementation tests. Leaves from soil-grown plants were hand-infiltrated with indicated bacterial suspensions (OD₆₀₀ = 0.0005), and bacterial titers were measured at 3 dpi. Bars represent SDs; *n* = 6 biologically independent samples from six individual T₁ lines. Different letters indicate significant differences (ANOVA, *P* < 0.05).

EDS1 protein stabilizes NPR1 protein in planta

In *Arabidopsis*, NPR1 protein is turned over by proteasome-mediated protein degradation and regulated by gene regulation (44, 51). We asked whether EDS1 regulates NPR1 at the transcriptional or translational level. In the dynamic expression assays, the basal and SA-induced NPR1 protein levels were significantly reduced in *eds1-2*, compared with wild-type control (Fig. 6D and fig. S6, C and E). However, *NPR1* transcript level in *eds1-2* was comparable to that in control plants (Fig. 6E and fig. S6D), albeit unexpectedly increased

at 3 hours after SA treatment. These results strongly indicate that EDS1 posttranscriptionally regulates NPR1. To investigate whether EDS1 stabilizes NPR1 protein, we compared protein levels of the constitutively expressed NPR1-GFP in *35S:NPR1-GFP/npr1-2* and *35S:NPR1-GFP/npr1-2;eds1-2* transgenic lines. In *35S:NPR1-GFP/npr1-2;eds1-2* plants, there were reduced levels of NPR1-GFP protein (Fig. 6F), which could be restored in the presence of the 26S proteasome inhibitor MG115 (Fig. 6F). We then analyzed NPR1 stability using cycloheximide (CHX), a potent protein synthesis inhibitor.

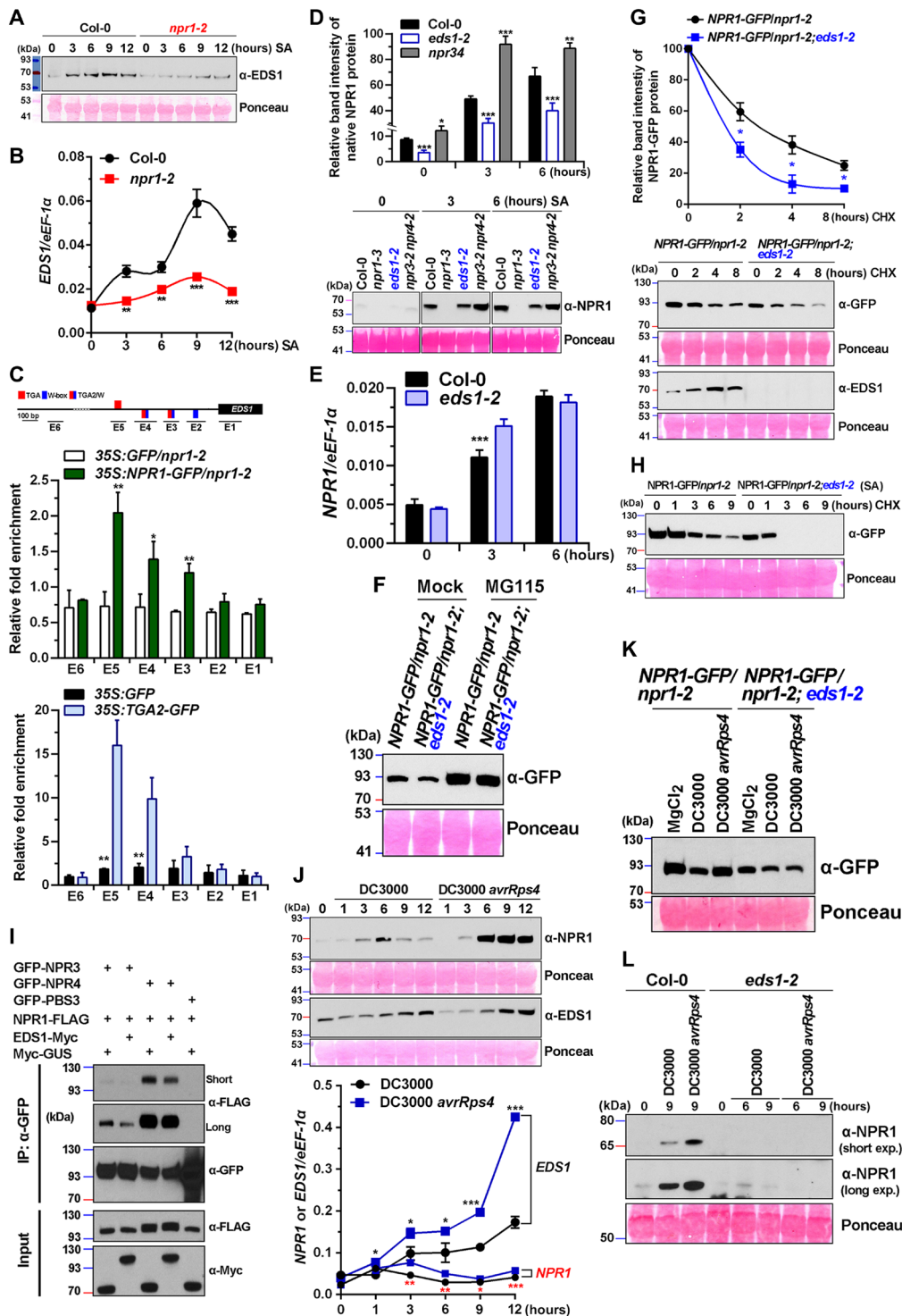


Fig. 6. A positive feedback loop of NPR1 and EDS1 in immune responses. (A to C) NPR1 transcriptionally regulates *EDS1*. The experiment was performed as in Fig. 4A (C). (D to H) EDS1 stabilizes NPR1. Transgenic seedlings were treated with 50 μ M MG115 or 0.2 mM CHX. Protein level of each genotype at start point was normalized to the same value [(G), top]. Note that EDS1 is induced by CHX probably through transcriptional induction and unknown mechanisms. Leaves were presprayed with 0.5 mM SA for 10 hours before hand infiltration with CHX (H). (I) *N. benthamiana* was cotransformed with constructs under the control of 35S promoter. (J to L) EDS1 protects NPR1 from degradation in plant-pathogen interactions. Protein [(J), top, and (L)] and mRNA [(J), bottom] levels in leaves from 4-week-old plants. Leaves were infiltrated with indicated pathogens (OD₆₀₀ = 0.01). Bars indicate (\pm) SDs; $n = 3$ biologically independent replicates (B and C). Bars indicate (\pm) SEs; $n = 3$ biologically independent experiments carried out on separate days (D, E, G, and J). Significances of differences from the control are shown for each time point or each applicon (t test, * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$). Protein (A and D) and mRNA (B and E) levels from seedlings treated with 0.5 mM SA. Total RNA was extracted and subjected to qPCR analysis. Total protein was analyzed by reducing SDS–polyacrylamide gel electrophoresis.

In a full CHX-chase assay, *eds1-2* mutation significantly accelerated the decay of uninduced NPR1-GFP protein (Fig. 6G) and rapidly promoted the decay of SA-induced NPR1-GFP (Fig. 6H). In contrast, *npr1-2* mutation did not affect CHX-resistant GFP-EDS1 or EDS1-eGFP protein (fig. S6, F and G). This is consistent with the above finding that NPR1 preferentially regulates *EDS1* transcription (Fig. 6, A to C). These results provide compelling evidence that EDS1 stabilizes NPR1 to maintain an optimal NPR1 threshold for immune responses.

The SA receptors NPR3 and NPR4 were proposed to be the adaptors of a Cullin3-based E3 ligase and promote the degradation of NPR1, JAZ (jasmonate ZIM-domain), and EDS1 (11, 55, 56). We examined the biochemical basis underlying the NPR1 homeostasis regulated by EDS1. In co-IP assays, GFP-NPR3 and GFP-NPR4 were efficiently coimmunoprecipitated with NPR1-FLAG under nonreducing (fig. S6H) and reducing conditions (Fig. 6I). However, expression of EDS1-Myc diminished the amount of NPR1-FLAG bound to GFP-NPR3/4 (Fig. 6I). Conversely, increased NPR3/4-eGFP was immunoprecipitated by NPR1 in the *eds1-2 npr3-2 npr4-2* (fig. S6I), compared to the immunoprecipitation in the *npr3-2 npr4-2* and the *npr1-2 npr3-2 npr4-2*. These suggest that EDS1 competes with NPR3/4 for NPR1 interaction, thereby stabilizing NPR1 in plants.

EDS1 stabilizes NPR1 during ETI to confer a robust defense

To further decipher the mechanism underlying the regulation of NPR1 protein stability in plant-pathogen interactions, we first investigated the accumulation of NPR1 protein in response to virulent and avirulent pathogen challenges in Col-0 plants. Time course expression analyses showed that infection with the avirulent *Pst* DC3000 *avrRps4* induced NPR1 protein more strongly than inoculation by the virulent *Pst* DC3000 (Fig. 6J, top), which obviously differs from the gene transcription patterns (Fig. 6J, bottom). These results demonstrated that NPR1 protein rather than its transcript hyperaccumulates during RRS1S/RPS4-mediated ETI, suggesting that ETI preserves NPR1.

Although ETI slightly enhanced the induction of *NPR1* transcription (Fig. 6J, bottom) to compensate for the degradation of NPR1 promoted by *Pst* DC3000 (Fig. 6, J and K), it is reasonable to speculate that ETI prevents NPR1 degradation mediated by the virulent pathogen. As anticipated, we found that the destruction of the constitutively expressed NPR1-GFP protein in *35S:NPR1-GFP/npr1-2* transgenic plants caused by *Pst* DC3000, as reported previously (31), was apparently restored by *Pst* DC3000 *avrRps4* (Fig. 6K), further supporting that AvrRps4-activated ETI prevents NPR1-GFP degradation mediated by virulence effector proteins. Moreover, the *eds1-2* mutation impeded the recovery of NPR1-GFP protein by *Pst* DC3000 *avrRps4* in *35S:NPR1-GFP/npr1-2;eds1-2* transgenic lines (Fig. 6K), firmly indicating that the prevention of NPR1-GFP degradation by ETI occurs in an EDS1-dependent manner. In addition, hyperaccumulated endogenous NPR1 protein protected by *Pst* DC3000 *avrRps4* was totally inhibited in the *eds1-2* mutant (Fig. 6L). Together, we conclude that EDS1 protects NPR1 from degradation in plant-pathogen interactions.

DISCUSSION

Genetic studies have identified several important positive regulators of plant immunity, including NPR1 (9), EDS1 (23), PAD4 (57), NDR1 (nonrace-specific disease resistance) (20), EDS5, PBS3 (GH3.12/*avrPphB* susceptible 3), and EPS1 (enhanced *Pseudomonas*

susceptibility) (58). Among them, the transcriptional regulator NPR1 has been known as the master regulator of SA signaling and SAR (8, 9). However, the mechanisms of NPR1-mediated transcriptional reprogramming are still poorly understood. EDS1 is required for plant basal defense, TNL-mediated ETI, and SAR (21, 23) and regulates the expression of a large number of defense-related genes (28, 29). Nonetheless, how EDS1 activates downstream defense genes remains obscure.

In the present study, we have shown the functionally physical and genetic interactions between two key immune regulators for the synergistical control of plant immune responses. The proposed model is illustrated in fig. S6J. We provide evidence suggesting that EDS1 acts as a transcriptional coactivator, which cooperates with NPR1 and Mediator in the transcription machinery for enhancing activation of defense genes upon immune induction. Interaction between two coactivators promotes direct recruitment of EDS1 onto promoters and influences the homeostasis of protein by stabilizing NPR1 under normal conditions and plant-pathogen interactions. We have elucidated an elaborate positive-feedback regulation of NPR1 and EDS1 by distinct mechanisms for amplifying defense responses.

EDS1 was classified as a lipase-like protein (23), but subsequent biochemical and structural studies showed that EDS1 has no lipase activity (26). Our study demonstrates that EDS1 serves as a transcriptional coactivator based on the following criteria. First, EDS1 binds chromatin regions in a stimulus-specific manner (Fig. 4, A and B, and fig. S4, A and B) and directly interacts with the transcriptional coactivator NPR1 (Fig. 1). Second, EDS1 has transactivation activity and contains two intrinsic TADs (Fig. 4C). We further found that acidic and hydrophobic amino acids are overrepresented within EDS1's two discrete TADs (Fig. 4C, bottom), indicating that EDS1 is a transcriptional activator with two AADs. In addition, EDS1 activates many defense genes in response to SA (Fig. 5C and figs. S3 and S4H). Furthermore, one TAD of EDS1 directly interacts with a subunit of the Mediator, CDK8 (Fig. 4, E and F), further supporting that EDS1 is a transcriptional activator that recruits Mediator in the transcription machinery. Since EDS1 itself likely does not bind chromosomal DNA directly (23), these findings support that EDS1 is a bona fide transcriptional coactivator. This is consistent with the notion that Mediator is recruited through a direct TAD of transcriptional (co)activator-Mediator interaction (49). It is worthwhile to mention that most previously reported AADs were identified in transcription activators with a DNA binding domain (45, 46, 48). Here, we have shown that EDS1, a transcriptional coactivator without a DNA binding domain, has two discrete AADs. Therefore, our study may shed light on the functions of transcriptional coactivators in general. Mechanistically, EDS1 is directly recruited to the specific SA-responsive cis-elements on *PR1* promoter by NPR1 and that it works together with NPR1, thus enhancing *PR1* transcription in SA-mediated defense (Fig. 5, A and B, and fig. S6J). Apart from associating with TGA motif-containing chromatin regions, EDS1 has also been shown to reside at the promoter regions containing W-box cis-elements in a similar manner to NPR1 (Fig. 4, A and B, and fig. S4, A and B). Consistently, several WRKY factors have been shown to interact with NPR1 (51, 59). Given that multiple WRKY factors exhibit intricate redundancy, cooperation, and antagonism on gene regulation and disease resistance to different pathogens (42, 43), the interaction of the NPR1-EDS1 complex with diverse TFs might fine-tune the dynamic gene expression for regulating plant growth and immune responses.

The interaction between NPR1 and the TGA2 subclass of TFs has been shown to play an important role in activating plant defense gene expression (17, 52). It is suggested that NPR1 is recruited by TGA2 onto the *PR1* promoter upon SA induction (15, 52), but whether NPR1 directly recruits transcriptional (co)factors to promote plant defense gene expression remains unknown. Our data indicate that NPR1 directly recruits a novel transcriptional coactivator EDS1 onto the *PR1* promoter via a physical interaction to stimulate *PR1* expression (Fig. 5, A to C). As the strong direct interaction between EDS1 and CDK8 was shown in this study, this suggests that Mediator can be recruited by NPR1-EDS1 complex to the *PR1* promoter in response to SA. Therefore, these findings indicate a novel prominent regulatory role of NPR1's transactivation, which is required for mediating the assembly of multiple regulatory (co)activators for specific transcriptional activation.

SA treatment activates the transactivation function of NPR1 presumably by releasing the autoinhibition of its cryptic transactivation activity (17). This suggests that NPR1 acts as a coactivator in an SA-dependent manner. Our study implies that the dynamic interaction of NPR1 with certain SA-induced immune regulators (e.g., EDS1) (fig. S1, D and E) may contribute to the relief of the repression of NPR1's transactivation activity by inducing conformation changes. Further structural and biochemical analyses of NPR1 and its partners are needed to test this possibility.

In mammalian systems, several studies have shown that diverse endogenous transcriptional activators form transcriptional condensates with the Mediator complex to robustly drive gene expression (60). Our study shows that two transcriptional coactivators form nuclear foci in plant cell nuclei and interact with a component of the Mediator, CDK8 (Figs. 1E and 4, E and F). Thus, these plant coactivators may form phase-separated nuclear condensates for active transcription. Most recently, NPR1 has been reported to facilitate the formation of cytoplasmic condensates for degradation of substrates to inhibit cell death (61). Nonetheless, nuclear NPR1 and EDS1 proteins may incorporate diverse transcriptional (co)factors into transcriptional activator concentrates for robust transcriptional reprogramming to relocate energy for defense instead of growth upon pathogen infection.

EDS1 can function in upstream of SA accumulation and regulate an SA-mediated defense pathway, as EDS1/PAD4-mediated signaling was previously shown to predominantly boost SA levels but not SA responsiveness (23, 37). Here, we further identified EDS1 as an essential positive regulator of SA signaling/response (downstream of SA) because it significantly facilitates expression of canonical SA marker genes (i.e., *PR1*, *PR2*, and *PR5*) in response to SA (Fig. 3A and fig. S3, A to C). In particular, EDS1-NPR1 synergistically accelerates SA signaling (Fig. 3C and fig. S3D) during transcriptional reprogramming and pathogen resistances (Figs. 2, B and C, and 3B). Accordingly, EDS1-mediated SA signaling contributes to RRS1S/RPS4-mediated ETI because constitutive overexpression of *EDS1* results in enhanced responsiveness to exogenous SA for protection against pathogen (62). To date, some *Arabidopsis* immune regulators [e.g., PAD4, AtELP2/3 (*Arabidopsis* elongator subunits 2/3), and MED14/16 (Mediator subunits 14/16)] have been well demonstrated to affect SA levels and/or signaling (39, 57, 63–65). Consequently, EDS1 modulates SA-mediated defense pathways involving activation of SA signaling transduction and upstream of SA accumulation, which is similar as the aforementioned accelerator of immune responses, AtELP2 (39). In comparison, NPR1, the master positive regulator of

SA signaling and SAR, suppresses SA accumulation (39, 63) to deliver a negative feedback in defense responses.

Multiple lines of evidence suggest that EDS1 also functions in SA-independent pathways during diverse immune responses (21, 28, 66). Genetic and transcriptomic data indicate a parallel action of EDS1-PAD4 and isochorismate synthase (ICS1)/SA induction-deficient 2 (SID2)-derived SA in apoplastic immunity (62, 67). In early TNL immunity, a follow-up study further revealed that EDS1-PAD4 interrupts MYC2-mediated suppression of an SA catabolism gene *BSMT1* (68), thereby reinforcing the SA-mediated defense antagonized by phytotoxin coronatine (COR)-stimulated jasmonic acid (JA) signaling (69), consistent with EDS1/PAD4 as negative regulators of JA pathway for defense against necrotrophic pathogens (70). In line with the notion that NPR1 is the master regulator of SA/SAR signaling, the induction of NPR1 protein (Fig. 6D and fig. S6C) and activation of NPR1 rely on different SA levels (11, 13, 14). Our study indicates that EDS1-NPR1 activates *PR* defense genes and other SA-induced genes in response to SA for rapid defense responses, proving that EDS1-NPR1 mediates potentiation of SA signaling for defense amplification. Similar to NPR1, EDS1 is essential for SAR signal generation and perception; EDS1-PAD4 is partially required for full extent of SAR (19, 21, 22). In a nutshell, EDS1-PAD4 prefers to act upstream of SA by antagonizing COR/JA MYC2 branch at early time points in ETI and basal immunity, whereas the chromatin-binding EDS1-NPR1 directly activates SA signaling and plays a major function at a later stage of local and systemic immunity. Overall, temporally and physically different EDS1-PAD4 and EDS1-NPR1 modules would be crucial for robust and sustained immune responses.

In plant-pathogen interactions, plants produce SA and JA in response to microbial pathogens and herbivorous insects. A reciprocal antagonism between SA- and JA-regulated transduction pathways plays key roles in activation of host defense responses (6). Cytosolic NPR1 has been reported to inhibit JA signaling by suppressing JA-responsive gene expression (71), while the aforementioned EDS1-PAD4 antagonizes MYC2-mediated JA signaling to bolster SA-mediated defense at early stages of RRS1S/RPS4-mediated ETI (69). It has also been described that SA/JA synergy is dependent on NPR1 and COI1 (coronatine insensitive 1) (72). In flg22-triggered PTI, JA-repressed *PAD4* not only contributes to antagonistic effects of JA on *EDS5*-derived SA accumulation but also is linked to JA/SA cooperation (73). At early stages of ETI and cell death, SA receptors such as NPR3/4 stimulate JA signaling and/or synthesis and interact with JAZ proteins to promote RPS2-mediated resistance (55). It seems that NPR1-EDS1 plays a role in the SA/JA cross-talk at certain stages of diverse defense responses. More studies are needed to further dissect the timing and mechanisms of SA/JA synergy and/or antagonism regulated by the interplay of NPR1 and EDS1.

This study shows that EDS1 rather than its classic partners (i.e., PAD4 and SAG101) has intrinsic transactivation activity (Fig. 4C and fig. S4G). As PAD4 and SAG101 are involved in the accumulation of EDS1 (74) and no direct interaction of NPR1 with PAD4 or SAG101 is detected (Fig. 1A and fig. S1A), it is suggested that the EDS1-PAD4 and/or EDS1-SAG101 complex may contribute to NPR1-mediated gene activation by preferentially bolstering EDS1 protein in plant-pathogen interactions. On the other hand, nucleocytoplasmic coordination of EDS1 and its interacting factors are involved in cell compartment-specific and full immune responses (25, 29). NPR1, EDS1, and PAD4 are localized in the cytosol and nucleus (13, 14, 74), while SAG101 is exclusively detected in the nucleus (74). It has been

suggested that neither SAG101 nor PAD4 affects nucleocytoplasmic localization of EDS1 (74). However, nucleocytoplasmic EDS1-PAD4 is required for signal transduction in basal immunity and SAR (21, 74); nuclear EDS1-SAG101 might be important for nuclear EDS1 retention (74). In our work, nucleocytoplasmic NPR1 does not affect the intracellular trafficking of EDS1 from cytoplasm to nucleus (fig. S5C). Instead, nuclear NPR1-EDS1 association is markedly enhanced in the specific nuclear compartment and, in turn, contributes to chromatin binding of EDS1 under induced states (Figs. 1, E and F, and 5A). Thus, the intricate dynamic association of EDS1 with its partners is essential for spatial and temporal coordination of full immune responses.

In summary, this work sheds light on the function of a transcriptional coactivator complex at the epicenter of plant immunity. Our study has revealed uncharacterized roles of NPR1 and EDS1 in signal transduction and activation of immune responses. Identification of EDS1 as a transcriptional coactivator not only opens an exciting avenue for studying the signaling pathways in plant immune responses but also sheds light on the molecular basis for general gene regulation. Meanwhile, direct recruitment of coactivator by NPR1 upon immune induction provides fresh insight into the mechanism of NPR1's transactivation.

MATERIALS AND METHODS

Plant materials and growth conditions

A. thaliana (L.) Heynh. seeds were sown on autoclaved soil and vernalized at 4°C for 3 days. Plants were germinated and grown in a growth chamber at 22°C day/20°C night with ~70% relative humidity and 12-hour light/12-hour dark photoperiod for middle-day conditions. To grow *Arabidopsis* seedlings in vitro, seeds were first sterilized by chlorine gas for 3 hours in a desiccator and sown on sterilized half-strength Murashige and Skoog (MS) media (pH 5.7) supplemented with 1% sucrose and 0.25% Phytigel with appropriate antibiotics. Plated seeds were stratified at 4°C for 3 days and then germinated in a growth chamber at 22°C day/20°C night under 16-hour light/8-hour dark photoperiod for long-day conditions. *N. benthamiana* was grown in a growth chamber at 25°C under middle-day conditions.

The *npr1-2* (8), *npr1-3* (8), *eds1-2* (28), *pad4-1* (57, 74), *rps4-2* (SALK_057697), and *npr3-2 npr4-2* (11) mutants are in the Columbia (Col-0) ecotype. The *eds1-1* is in the Wassilewskija (Ws-0) ecotype (22, 23).

Constructs, transgenic plants, and genetic analysis

For generating expression constructs, the Gateway Cloning Technology (Invitrogen) and In-Fusion Advantage PCR Cloning Kit (Clontech) were used. Most DNA fragments were amplified and cloned into entry vectors such as pDONR207 and pENTR/D-TOPO (Invitrogen) and then transferred to the destination vectors. The binary vectors were transformed into *Agrobacterium* by electroporation and then transformed into *N. benthamiana* or *Arabidopsis* lines. The stable T₂ transgenic lines with single inserts were analyzed and carried to produce T₃ homozygous progenies. At least two independent homozygous lines expressing target protein significantly were selected for further studies. The transgenic plants used in all experiment are homozygous lines, unless specifically mentioned otherwise. The sequence-specific primers (table S1) and recombinant DNA constructs (table S2) for all experiments are listed and described previously (31).

To create pEDS1:EDS1-FLAG expression clones, the genomic coding region and 2-kb upstream sequences of EDS1 DNA were cloned into entry clone and then transferred to the pEarleyGate302-3xFLAG destination vector provided by X. Zhong (University of Wisconsin-Madison); the combined binary vector was introduced into *Arabidopsis eds1-2* mutant background to obtain the pEDS1:EDS1-FLAG/*eds1-2* transgenic lines. Similarly, the pNPR1-NPR1-9xMyc/*npr1-2* transgenic lines were obtained. For 35S:EDS1-eGFP/*eds1-2* and 35S:GFP-EDS1/*eds1-2* transgenic lines, the full-length EDS1 cDNA was cloned into entry vector and transferred into pK7FWG2 and pMDC43 destination vector; these binary plasmids were introduced into *eds1-2* plants, respectively. To generate 35S:GFP-NPR1 over-expression transgenic lines and 35S:NPR1-GFP/*npr1-2* plants, full-length NPR1 cDNA was cloned into pMDC43 destination vector and pCB302 binary vector and then the resulting vectors were introduced into Col-0 wild-type and *npr1-2* mutant backgrounds, respectively.

All crosses among different genotypes were performed by pollinating the emasculated flowers of maternal recipient with pollen from male donor. The *npr1-2 eds1-2* and *eds1-2 npr1-2* double mutants were generated by crossing female *npr1-2* with *eds1-2* and by crossing female *eds1-2* with *npr1-2*, respectively. The *eds1-2 npr3-2 npr4-2* and *npr1-2 npr3-2 npr4-2* triple mutants were obtained by crossing *npr3-2 npr4-2* with *eds1-2* and *npr1-2*, respectively, and genotyping with specific primers (table S1).

To generate 35S:EDS1-eGFP/*eds1-2;npr1-2* lines, *npr1-2* was crossed with 35S:EDS1-eGFP/*eds1-2* plants. For 35S:NPR1-GFP/*npr1-2;eds1-2* lines, 35S:NPR1-GFP/*npr1-2* as a recipient was crossed with *eds1-2*. The double mutations in the segregating F₂ populations was identified by a *npr1-2* cleaved amplified polymorphic sequence (CAPS) marker and by PCR using primers flanking the *eds1-2* deletion region (table S1); the homozygosity for the EDS1-eGFP or NPR1-GFP transgene was confirmed in the next generation by genotyping using specific primers for eGFP or GFP (table S1). All the successive plants and controls at the same generation were selected in further studies. To generate pNPR1:Myc-NPR1 plants containing pEDS1:EDS1-FLAG, the pEDS1:EDS1-FLAG/*eds1-2* transgenic plants were crossed with pNPR1:Myc-NPR1/*npr1-3* plants (65) provided by Z. Mou (University of Florida). The F₂ plants were selected on antibiotics and genotyped using *eds1-2* and *npr1-3* CAPS marker (39) and specific primers for transgene in next generations.

Y2H and yeast monohybrid assays

Y2H assays were performed as described previously (31). The pDEST-GBKT7-based bait vectors were transformed into the yeast strain Y187, and the yeast strain AH109 was transformed with pDEST-GADT7-based vectors. The fresh diploids by yeast mating were used to detect protein-protein interactions on selective triple dropout media (without Leu, Trp, and His) plus 1 mM 3-aminotriazole and quadruple dropout medium (without Leu, Trp, His, and Ade).

For yeast monohybrid assays (45), pDEST-GBKT7-based GAL4 DNA-BD fusion vectors were transformed into the yeast strain AH109 including several reporters (*HIS3*, *MEL1*, and *lacZ*) under distinct GAL4 upstream activating sequences as described in Matchmaker GAL4 Two-Hybrid System 3 & Libraries User Manual (Clontech). The transformants were grown on synthetic dropout agar medium lacking Trp and His (-WH) and detected on synthetic dropout/-WH/X- α -Gal (Biosynth). The liquid cultures of yeast cells were used to detect the *lacZ* expression in quantitative

β -galactosidase assays with *o*-nitrophenyl- β -D-galactopyranoside (Amresco) performed according to the Yeast Protocols Handbook (Clontech).

Pull-down assay

The recombinant protein expression and in vitro pull-down assay were carried out as previously described with minor modification (31). For GST-fusion protein expression, the coding sequences of β -glucuronidase (GUS), EDS1, and PAD4 were cloned into entry clone and transferred into pDEST15, respectively. These GST-fusion constructs and GST (empty pGEX-4T-1 vector) were heterologously expressed in the *Escherichia coli* Rosetta (DE3) cell line. The Trx-His₆-NPR1 protein was expressed in *E. coli* OverExpress C41 (DE3) strain using the plasmid pET-32a. For the pull-down assay, Trx-His₆-NPR1 protein in 2 ml of extracts was immobilized on 30 μ l of Ni-nitrilotriacetic acid agarose at 4°C for 1 hour. After washing several times, the whole-cell extract of GST-protein fusion was added to each immobilized sample for 1 hour at 4°C. After washing, the bound proteins were eluted by boiling in sample buffer and subjected to immunoblotting analysis. The signals were visualized as described previously.

Agrobacterium-mediated transient expression

Agrobacterium-mediated transient expression in *N. benthamiana* leaves were performed as described previously (31). *Agrobacterium tumefaciens* strain (GV3101/pMP90) carrying the indicated constructs were used together with the p19 strain for infiltration of *N. benthamiana* leaves using a needleless syringe.

The transient assays in *Arabidopsis* seedlings were carried out using a highly efficient and robust method, named AGROBEST (75). The 7-day-old seedlings grown on soil under middle-day conditions were incubated with the disarmed *Agrobacterium* (GV3101/pMP90) preinduced by 200 μ M acetosyringone. Three days after infection, seedlings were transferred to fresh 1/2 MS liquid medium [1/2 MS, 0.05% MES, and 0.5% sucrose (pH 5.5) with KOH] containing 100 μ M Timentin for another 2 days.

BiLC and BiFC assays

Agrobacterium-mediated transient expressions in *N. benthamiana* leaves were performed as described previously (31). For BiLC assay, the full-length coding sequence of target gene was fused to the N or C terminus of firefly luciferase using pCAMBIA1300 nLUC or pCAMBIA1300 cLUC vector; STG1a-nLUC and cLUC-RAR1 constructs were used as positive interaction control (76). Leaves excised 2 days after transient expression were sprayed with luciferin solution (100 μ M luciferin and 0.01% Triton X-100) and kept in the dark for 2 hours to quench fluorescence. Luc activity was observed with a low-light cooled charge-coupled device imaging apparatus (Andor iXon). In BiFC assay, the relative entry clone was transferred into pMDC43-nVenus and pMDC43-cCFP vectors (24) provided by W. Gassmann (University of Missouri). The leaf tissues from the infiltrated area were observed under a confocal microscope (Leica TCS SP8) with the VENUS/GFP filters: 488-nm excitation and 530-nm emission.

Co-IP and immunoblotting assays

Protein fractionations for immunoblotting and co-IP assays in *N. benthamiana* and *Arabidopsis* were performed as previously described (31). The homogenate was sonicated on ice and optionally treated with Benzonase Nuclease (MilliporeSigma) for 30 min on

ice. The solution was filtered through Miracloth (Calbiochem) or syringe filters. The Myc-Trap_MA (ChromoTek), anti-FLAG M2 magnetic beads (Sigma-Aldrich), and GFP-Trap_MA (ChromoTek) were used to immunoprecipitate the protein complexes. Pierce Protein A/G magnetic beads (Thermo Fisher Scientific) were used for antibody affinity binding. Immunoblotting was performed with anti-Myc Tag (Thermo Fisher Scientific), anti-FLAG M2 antibodies (Sigma-Aldrich), and anti-GFP antibodies (Clontech).

Pathogen growth assays

Inoculation of plants with pathogens and pathogenicity tests were performed as described previously (31). Three full-grown leaves on each 4- to 6-week-old plant grown under middle-day conditions were inoculated with different *Pseudomonas* strains. The three leaf discs from an individual plant were pooled for each sample, and six such replicates were used for each genotype in pathogen growth assay.

Real-time qPCR

Gene expression analysis by qPCR was carried out as previously described with minor modification (31). Total RNA was extracted using RNazol RT (Sigma-Aldrich), and 2 μ g of total RNA was subjected to reverse transcription using the qScript cDNA Synthesis Kit (Quanta). Real-time PCR was performed using PerfeCTa SYBR Green FastMix (Quanta). Expression was normalized against constitutively expressed *eEF-1 α* . The primers used for qPCR in this study are shown in table S1.

Cell fractionation

Preparation of nuclear and cytoplasmic fractions was performed according to the user manual supplied with the CellLytic PN Plant Nuclei Isolation/Extraction Kit (Sigma-Aldrich) with minor modifications. Approximately 2 g of plant tissues was suspended in nuclei isolation buffer (NIB) and passed through a provided filter mesh. After centrifugation for 15 min, the supernatant was used for further extraction of cytoplasmic proteins, and the pellet was used to further extract nuclei and nuclear proteins. The transferred supernatant was centrifuged for 10 min at 12,000 rpm, 4°C, and the clean supernatant was collected as cytoplasmic fractions. The initial pelleted nuclei were resuspended in 10 ml of NIBA [1 \times NIB, 1 mM dithiothreitol (DTT), 1 \times protease inhibitor cocktail, and 0.5% Triton X-100]. After centrifugation, isolation of nuclei was carried out as described with Semi-pure Preparation of Nuclei Procedures based on the manufacturer's protocol. The cellular fractions were analyzed on reducing SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Phosphoenolpyruvate carboxylase (PEPC) and ribulose-1,5-bisphosphate carboxylase-oxygenase (RuBisCo) were detected and used as cytoplasmic markers, and histone H3 was used as a nuclear marker.

ChIP analysis

ChIP was performed according to a previous report with modifications (64). Approximately 3 g of 4-week-old soil-grown plants or 3-week-old seedlings were harvested and vacuum-infiltrated with 1% formaldehyde for cross-linking. The cross-linking reaction was subsequently stopped by 150 mM glycine. Samples were washed three times with sterile deionized water, dried on paper towel, frozen, and stored at -80°C for further use. For chromatin isolation, plant tissues were ground to a fine powder in liquid nitrogen and mixed with 30 ml of cold NIB [0.25 M sucrose, 15 mM Pipes (pH 6.8) or

10 mM tris-HCl (pH 7.5), 5 mM MgCl₂, 60 mM KCl, 15 mM NaCl, 1 mM CaCl₂, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), pepstatin A (2 µg/ml), aprotinin (2 µg/ml), and 1 mM DTT]. Samples were incubated on ice for 5 min with gentle vortex and then filtered through two layers of Miracloth (Calbiochem) and centrifuged at 4°C at 3000g for 20 min. The nuclear pellets were gently resuspended in 1.5 ml of cold nuclei lysis buffer [50 mM tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.3% sarkosyl, 1% Triton X-100, 50 µM MG-115, 1 mM PMSF, protease inhibitor cocktail, and 1 mM DTT] and incubated on ice with gentle mixing for 5 min. Chromatin was sheared into approximate 500–base pair DNA fragments using M220 Focused-ultrasonicator (Covaris) and centrifuged at 13,000g at 4°C for 15 min. The supernatant was collected for further steps.

For the immunoprecipitation step, the samples were diluted with ChIP dilution buffer [20 mM tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 50 µM MG-115, 1 mM PMSF, protease inhibitor cocktail, and 1 mM DTT] and precleared for 1 hour using control magnetic agarose beads blocked with bovine serum albumin (100 µg/µl). After removing the beads, 5% of precleared chromatin was retained as input control. Meanwhile, the remaining samples were mixed with conjugated anti-Myc tag antibody (Abcam) with Magna ChIP Protein A Magnetic Beads (Sigma-Aldrich) for Myc-NPR1, anti-FLAG M2 magnetic beads (Sigma-Aldrich) for FLAG-EDS1, or GFP-Trap_MA beads (ChromoTek) for EDS1-eGFP. The mixture was incubated at 4°C for 4 hours with gentle rotation, and then, the immunocomplexes were washed twice each with low salt, high salt, LiCl, and tris-EDTA buffer.

In the reverse cross-linking steps, ChIP sample and input control were mixed with 20% Chelex 100 Resin (Sigma-Aldrich) solution at room temperature and incubated for 10 min at 95°C shaking every 3 min. Once the sample was cooled down, 20 µg of proteinase K (Invitrogen) was added to a final volume of 200 µl of ChIP reaction in TE and incubated at 50°C for 1 hour, followed by boiling for 10 min. After spin down, the supernatant was transferred and retained, and the pelleted beads were washed with TE; the washing flow-through was added to the initial supernatant. Then, 5 µg of ribonuclease A (Thermo Scientific) was added into each sample and incubated at 37°C for 30 min. Immunoprecipitated DNA was purified using a mixture of phenol:chloroform:isoamyl alcohol (25:24:1) followed by chloroform extraction, ethanol precipitated using Dr. GentLE Precipitation Carrier (TaKaRa) with incubation at –80°C for 1 hour, recovered by centrifugation, washed, and resuspended in 100 µl of TE.

Recovered DNA was quantified by qPCR described as above, with the locus-specific primers (table S1), and ChIP-qPCR was performed with at least three technical replicates. Relative DNA level for each amplicon was calculated against the total input using the $\Delta\Delta C_{T_T}$ method. Relative fold enrichment was standardized to the *Actin2* open reading frame.

Antibodies

Primary antibodies used in this study are as follows: mouse monoclonal anti-GFP (JL-8; Clontech, ab2313808), mouse monoclonal anti-FLAG M2 (Sigma-Aldrich, ab439702), mouse monoclonal anti-FLAG M2-peroxidase [horseradish peroxidase (HRP)] (Sigma-Aldrich, ab439702), rat monoclonal anti-HA (3F10; Roche, ab390917), mouse monoclonal anti-GST (GenScript, ab771432), mouse monoclonal anti-His (GenScript, ab914704), mouse monoclonal anti–c-Myc tag

(Thermo Fisher Scientific, ab2556560), rabbit polyclonal anti-PEPC (Agrisera, ab2063166), rabbit polyclonal anti–histone H3 (Agrisera, AS10710), rabbit polyclonal anti-NPR1 (Agrisera, AS121854), rabbit polyclonal anti-PR1 (Agrisera, AS10687), rabbit polyclonal anti-EDS1 (Agrisera, AS132751), goat anti-mouse immunoglobulin G (IgG) (HRP) (Thermo Fisher Scientific, catalog no. G-21040), goat anti-rabbit IgG (HRP) (Thermo Fisher Scientific, catalog no. G-21234), and goat anti-rat IgG (HRP) (Thermo Fisher Scientific, catalog no. 31470).

Quantification and statistical analysis

The results of Western blots were quantified with software ImageJ (National Institutes of Health). Statistical analysis was conducted with the software of GraphPad Prism 6.0 using one-way or two-way analysis of variance (ANOVA) with Tukey’s multiple comparisons test or using multiple Student’s *t* tests. Error bars represent SDs or SEs. Statistically significant differences are marked with asterisks (two-tailed Student’s *t* test, **P* < 0.05, ***P* < 0.01, and ****P* < 0.001) or different letters (*P* < 0.05). For instance, different letters (a, b, c, etc.) are used to label samples with statistical differences, whereas the “abc” is used to mark samples with no statistical difference to other samples labeled with “a,” “b,” or “c.” Detail statistical differences can be found in the figures and figure legends.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at <https://science.org/doi/10.1126/sciadv.abl7173>

[View/request a protocol for this paper from Bio-protocol.](#)

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