The Plant Cell, Vol. 21: 2527–2540, August 2009, www.plantcell.org

ETHYLENE INSENSITIVE3 and ETHYLENE INSENSITIVE3-LIKE1 Repress *SALICYLIC ACID INDUCTION DEFICIENT2* Expression to Negatively Regulate Plant Innate Immunity in *Arabidopsis* [W][OA]

Huamin Chen,[a,b,1,2] Li Xue,[b,c,2] Satya Chintamanani,[d,2] Hugo Germain,[e] Huiqiong Lin,[b] Haitao Cui,[b,c] Run Cai,[a] Jianru Zuo,[c] Xiaoyan Tang,[d] Xin Li,[e] Hongwei Guo,[f] and Jian-Min Zhou[b,3]

[a] School of Agriculture and Biology, Shanghai Jiaotong University, Shanghai 200240, China
[b] National Institute of Biological Sciences, Beijing 102206, China
[c] State Key Laboratory of Plant Genomics and National Plant Gene Research Center, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing 100101, China
[d] Department of Plant Pathology, Kansas State University, Manhattan, Kansas 66506
[e] Michael Smith Laboratories, University of British Columbia, Vancouver, BC V6T 1Z4, Canada
[f] National Laboratory of Protein Engineering and Plant Genetic Engineering, College of Life Sciences, Peking University, Beijing 100871, China

Pathogen/microbe-associated molecular patterns (PAMPs/MAMPs) trigger plant immunity that forms the first line inducible defenses in plants. The regulatory mechanism of MAMP-triggered immunity, however, is poorly understood. Here, we show that *Arabidopsis thaliana* transcription factors ETHYLENE INSENSITIVE3 (EIN3) and ETHYLENE INSENSITIVE3-LIKE1 (EIL1), previously known to mediate ethylene signaling, also negatively regulate PAMP-triggered immunity. Plants lacking EIN3 and EIL1 display enhanced PAMP defenses and heightened resistance to *Pseudomonas syringae* bacteria. Conversely, plants overaccumulating EIN3 are compromised in PAMP defenses and exhibit enhanced disease susceptibility to *Pseudomonas syringae.* Microarray analysis revealed that EIN3 and EIL1 negatively control PAMP response genes. Further analyses indicated that *SALICYLIC ACID INDUCTION DEFICIENT2* (*SID2*), which encodes isochorismate synthase required for pathogen-induced biosynthesis of salicylic acid (SA), is a key target of EIN3 and EIL1. Consistent with this, the *ein3-1 eil1-1* double mutant constitutively accumulates SA in the absence of pathogen attack, and a mutation in *SID2* restores normal susceptibility in the *ein3 eil1* double mutant. EIN3 can specifically bind *SID2* promoter sequence in vitro and in vivo. Taken together, our data provide evidence that EIN3/EIL1 directly target *SID2* to downregulate PAMP defenses.

INTRODUCTION

Plant innate immunity is activated upon the recognition of pathogen/microbe-associated molecular patterns (PAMPs/MAMPs) by surface-localized immune receptors or stimulation of cytoplasmic immune receptors by pathogen effector proteins (Ausubel, 2005; Jones and Dangl, 2006). PAMP-triggered immunity (PTI) is central to plant resistance to numerous potential pathogens and is thus indispensable for plant survival in the environment (Chisholm et al., 2006).

The PTI signal transduction pathway is not well understood. The best understood PTI pathway is mediated by FLS2, the *Arabidopsis thaliana* receptor for bacterial flagellar peptide flg22 (Schwessinger and Zipfel, 2008). The binding of flg22 induces the association of FLS2 with BAK1, a receptor-like kinase. This ligand-induced oligomerization activates the FLS2 kinase, which subsequently activates cytoplasmic signaling pathways. Downstream, two mitogen-activated protein (MAP) kinase cascades are rapidly activated to regulate defenses (Bittel and Robatzek, 2007). MEKK1, MKK1/MKK2, and MPK4 constitute a MAP kinase cascade that negatively regulates PTI defenses. The *mekk1* mutant, *mkk1 mkk2* double mutant, and *mpk4* mutant all display constitutive defenses (Petersen et al., 2000; Ichimura et al., 2006; Qiu et al., 2008). MPK3 and MPK6, two related MAP kinases, are thought to positively regulate PTI defenses, but genetic demonstration of their function is hampered by the lethality of the *mpk3 mpk6* double mutant (Bittel and Robatzek, 2007).

Salicylic acid (SA) is a major plant defense hormone central to the activation of a range of defenses including the induction of pathogenesis-related (PR) genes, systemic acquired resistance,

[1] Current address: State Key Laboratory for Biology of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing 100193.
[2] These authors contributed equally to this work.
[3] Address correspondence to zhoujianmin@nibs.ac.cn.
The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Jian-Min Zhou (zhoujianmin@nibs.ac.cn).
[W]Online version contains Web-only data.
[OA]Open access articles can be viewed online without a subscription.
www.plantcell.org/cgi/doi/10.1105/tpc.108.065193

and hypersensitive response (Durrant and Dong, 2004). Recent data indicate that SA is also required for the full activation of PTI (Mishina and Zeier, 2007; Tsuda et al., 2008). Genetic and biochemical studies in the last 15 years have led to a comprehensive understanding of the signaling mechanism underlying SA-mediated disease resistance. NPR1 plays a central role in SA-dependent disease resistance. The conformation of the NPR1 protein is regulated by cellular redox state, enabling SA-induced entry of NPR1 into the nucleus (Mou et al., 2003; Tada et al., 2008). The nuclear entry and function of NPR1 are also regulated by phosphorylation and ubiquitination (Spoel et al., 2009). NPR1 interacts with the TGA class transcription activators and activates the transcription of a number of defense genes. In addition, PAD4 and EDS1 function to amplify the SA defenses by a positive feedback loop (Feys et al., 2001).

Contrary to our extensive knowledge concerning SA-mediated signal transduction, little is known about the control of SA biosynthesis. The biosynthesis of SA is strongly induced upon pathogen infection. This pathogen-induced SA biosynthesis is controlled by *SID2*, which encodes isochorismate synthase 1 (ICS1; Wildermuth et al., 2001). *Arabidopsis sid2* mutants are defective in pathogen-induced SA accumulation and are severely compromised in disease resistance to biotrophic pathogens (Nawrath and Metraux, 1999; Wildermuth et al., 2001). Thus, the regulation of *SID2* expression is fundamental to plant immunity.

Here, we show that ETHYLENE INSENSITIVE3 (EIN3) and ETHYLENE INSENSITIVE3-LIKE1 (EIL1), two closely related *Arabidopsis* transcription factors previously known to regulate the ethylene pathway, negatively regulate *SID2* expression and SA biosynthesis to repress plant immunity. The *ein3 eil1*double mutants constitutively expressed *SID2* and a large number of PAMP response genes, overaccumulate SA, and showed increased disease resistance to *Pseudomonas syringae* bacteria. The enhanced resistance and defense gene expression were abolished in the *ein3 eil1 sid2* triple mutant. Conversely, plants that overaccumulate EIN3 protein display enhanced susceptibility to *P. syringae* bacteria. The *SID2* promoter-*LUC* reporter gene showed greatly increased activity in *ein3 eil1* mutant protoplasts. Moreover, the EIN3 protein was capable of binding to the *SID2* promoter. These results uncover a role for EIN3 and EIL1 in the crosstalk of ethylene and SA signaling pathways.

RESULTS

The *rrb6* Mutant Is a Novel *ein3* Allele

We previously showed that a jasmonate (JA) and ethylene (ET) response gene, *RAP2.6*, which encodes an ET response factor, is transcriptionally activated by *P. syringae* effectors, including AvrB (He et al., 2004). To identify *Arabidopsis* mutants with altered responses to *P. syringae* bacteria, we developed a *RAP2.6-LUC* promoter reporter-based mutant population and screened for mutants displaying reduced reporter activity in response to *P. syringae* DC3000 (*avrB*) bacterial infection (Shang et al., 2006). One of these mutants, *rrb6* (for reduced responsiveness to *avrB*) displayed reduced reporter gene expression when infiltrated with the virulent strain DC3000, incompatible strain DC3000 (*avrB*), and water (Figures 1A and 1B), indicating that the mutant was nonspecifically affected in biotic and abiotic responses. Because the *RAP2.6* reporter gene is regulated by phytohormones JA and ET, we tested if the *rrb6* mutant was affected in ET signaling. Wild-type *Arabidopsis* seedlings treated with 1-aminocyclopropane-1-carboxylate (ACC), a precursor that can be rapidly converted to ET by plants, showed inhibition of root and hypocotyl elongation, exaggerated tightening of the apical hook, and the swelling of hypocotyl collectively called the triple response (Guzmán and Ecker, 1990). *rrb6* showed a uniform insensitive phenotype at 10 μM ACC (Figure 1C). In the absence of ACC, the mutant seedlings were similar to the wild type. The genetic nature of the *rrb6* mutation was determined by crossing the homozygous mutant plants with the wild-type transgenic plants carrying *RAP2.6-LUC*. The F1 seedlings showed a normal ET response, indicating that *rrb6* is a recessive mutation. The morphology of *rrb6* plants was quite similar to that of the wild type, with the exception that the leaves are slightly larger.

The *rrb6* mutation was initially mapped to the top arm of chromosome III between the simple sequence length polymorphism markers MSJ11 and MLM2. The size of *rrb6* F2 population was then increased to fine-map the mutation in a region of ~100 kb between the markers MQC12 and MOE17 (see Supplemental Table 3 online). Because *rrb6* is ET insensitive and was mapped to the region containing *EIN3*, which is a known ET signaling gene, we tested whether *rrb6* is an *ein3* allele. Sequence analysis identified a point mutation in *rrb6* at nucleotide 1599 of *EIN3* (C to T), which resulted in a P216S substitution in the Pro-rich domain. The previously reported allele *ein3-1* has a mutation at nucleotide 1598 (G to A) to introduce a stop codon at amino acid 215 (Chao et al., 1997). To further confirm that the ET-insensitive phenotype in *rrb6* is due to the mutation in the *EIN3* gene, we crossed the *rrb6* mutant with *ein3-1* plants. As shown in Supplemental Table 1 online, F1 plants were completely insensitive to ET. Several alleles of *ein3* have been discovered to date (Chao et al., 1997; Alonso et al., 2003), and we renamed *rrb6* as *ein3-4*.

EIN3 and EIL1 Negatively Regulate Disease Resistance to *P. syringae*

EIN3 and its close homolog EIL1 belong to a family of transcription factors known to regulate the ET response. We sought to determine the role of EIN3 and EIL1 in disease resistance. We inoculated *ein3-1*, *eil1-1*, and the *ein3-1 eil1-1* double mutant with *P. syringae* DC3000 bacteria. The *ein3-1 eil1-1* double mutant was consistently more resistant than Columbia-0 (Col-0) in multiple experiments and supported ~10-fold less bacterial growth at 2 and 4 d after inoculation (Figure 2A). The *ein3-1* and *eil1-1* mutants showed slightly enhanced resistance compared with Col-0 at 4 d after inoculation in this particular experiment, but the results were variable in other experiments. We also inoculated the *ein3-1* and *ein3-1 eil1-1* mutants with DC3000 carrying *avrRpt2*, which conditions *RPS2*-specific resistance in *Arabidopsis* plants. Figure 2B shows that the *ein3-1 eil1-1* double mutant displayed enhanced resistance to this bacterium.

To further test the role of *EIN3* in *P. syringae* resistance, we inoculated an *EIN3* overexpression line with DC3000. This line carried a 35S:EIN3 transgene and displays a dwarf phenotype

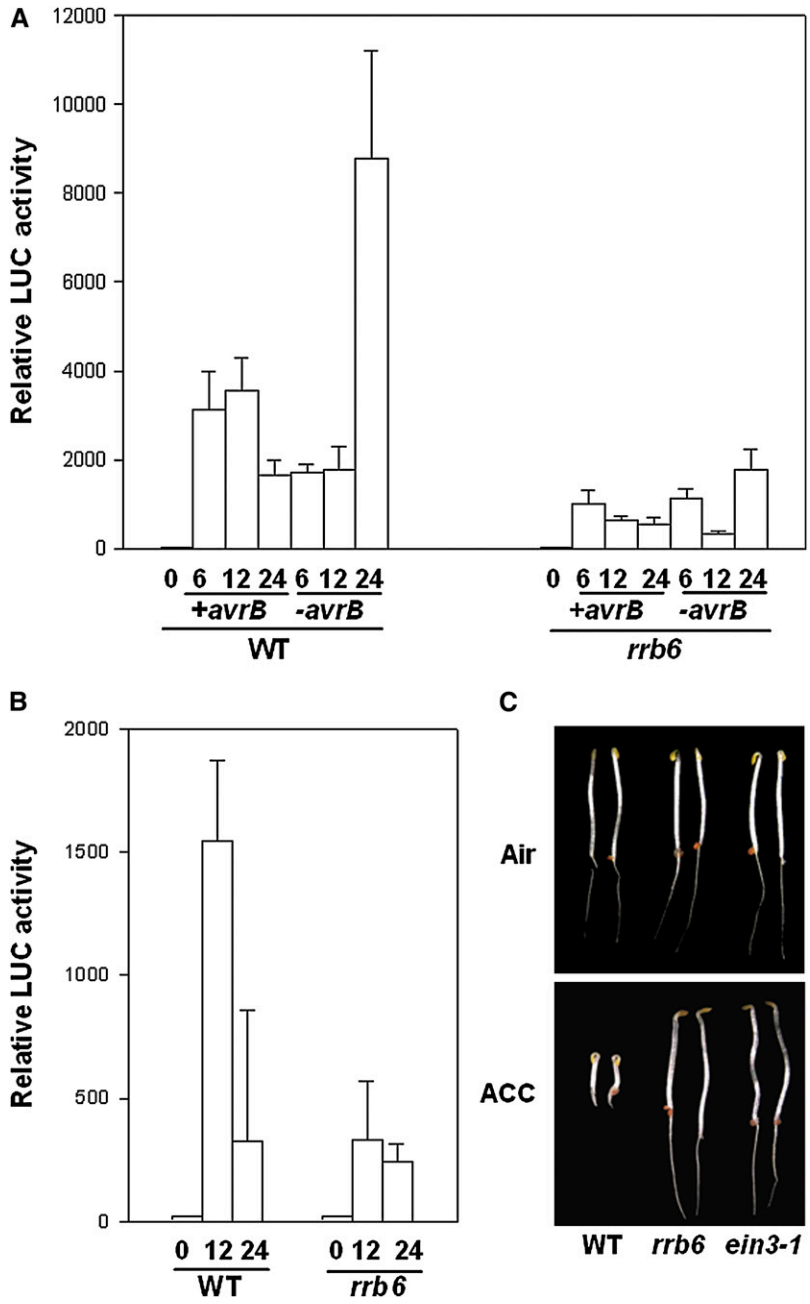

Figure 1. *rrb6* Displays Reduced Expression of *RAP2.6-LUC* Reporter and Defects in Triple Response.

(A) *RAP2.6-LUC* reporter activity in wild-type and *rrb6* mutant leaves infiltrated with DC3000 bacteria with or without *avrB.*
(B) *RAP2.6-LUC* reporter activity in leaves infiltrated with water. Note that the scale is different compared with that in **(A)** because water treatment induces lower reporter activity than bacteria treatment.
(C) Triple response of 3-d-old wild-type and *rrb6* seedlings in the presence or absence of ACC. The *ein3-1* mutant was included as a control.
Each data point in **(A)** and **(B)** consisted of at least three leaves. Error bars indicate SD.

indicative of constitutive ET response (Solano et al., 1998). Figure 2C shows that the *EIN3* overexpression line was more susceptible to DC3000 and supported ~10-fold more bacterial growth than did the Col-0.

We also inoculated the *ein3-4* mutant with DC3000 and determined bacterial growth. To our surprise, the *ein3-4* mutant was consistently more susceptible than the wild type to DC3000 in multiple experiments. DC3000 bacteria grew ~10-fold more in *ein3-4* than in the wild type (Figure 2D). The *ein3-4* mutant plants also developed more severe disease symptoms than the wild type (see Supplemental Figure 1 online). To further determine the effect of *ein3-4* mutation on disease resistance, we inoculated

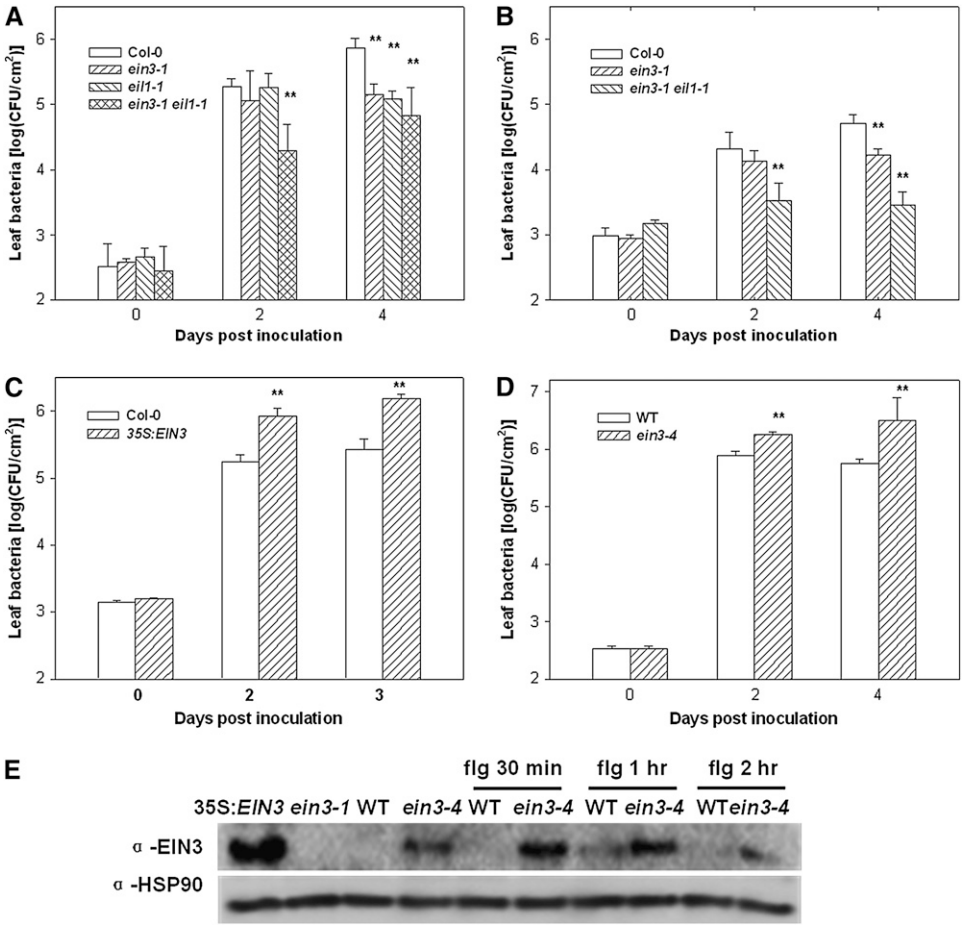

Figure 2. *EIN3* and *EIL1* Negatively Regulate Resistance to *P. syringae* Bacteria.

(A) *ein3-1 eil1-1* exhibits enhanced resistance to DC3000. Plants of the indicated genotypes were inoculated with DC3000 bacteria, and bacterial populations in the leaf were determined at the indicated times.

(B) *ein3-1 eil1-1* shows enhanced resistance to DC3000 (*avrRpt2*). Plants of the indicated genotypes were inoculated with DC3000 (*avrRpt2*) bacteria, and bacterial populations in the leaf were determined at the indicated times.

(C) *EIN3* overexpression enhances resistance to DC3000. Plants of the indicated genotypes were inoculated with DC3000 bacteria, and bacterial populations in the leaf were determined at the indicated times.

(D) *ein3-4* enhances susceptibility to DC3000. Wild-type *RAP2.6-LUC* transgenic plants were used as a control.

(E) *ein3-4* enhances EIN3 protein stability. Four-day-old etiolated seedlings were treated with 1 μM flg22 for the indicated times and examined by immunoblot using anti-EIN3 antibodies or anti-HSP90 antibodies (for loading control). EIN3 protein exists at a low level in wild-type *RAP2.6-LUC* transgenic seedlings and was not detected in this blot. The 35S:*EIN3* and *ein3-1* seedlings were used as positive and negative controls, respectively. Each data point consisted of at least three samples. Error bars indicate SD. * and ** indicate significant difference at 0.05 and 0.01, respectively, between mutants and Col-0 or wild-type *RAP2.6-LUC* plants at the same time point (Student's *t* test). Results shown are a representative of three (**[A]** to **[C]**) or five **(D)** independent experiments.

wild-type, *ein3-4*, and *ein3-4* × wild-type F1 plants with DC3000 (*avrB*) bacteria. Supplemental Figure 2 online shows that the *ein3-4* acted semidominantly in the suppression of disease resistance. An examination of the EIN3 protein level showed that the EIN3 protein accumulated to a high level in the *ein3-4* mutant plants, indicating that the *ein3-4* mutation resulted in greater stability of the EIN3 protein (Figure 2E). Interestingly, flg22 induced the accumulation of EIN3 protein in both Col-0 and *ein3-4* seedlings 1 h after treatment. These results indicated that the $EIN3^{P216S}$ mutant protein encoded by *ein3-4* was fully functional in repressing plant disease resistance, although it was completely nonfunctional in regulating the ET induced triple response. Together, these results demonstrated that EIN3 and EIL1 negatively regulate resistance to both compatible and incompatible *P. syringae* bacteria in *Arabidopsis*.

EIN3 and EIL1 Are Negative Regulators of PTI Defenses

The results described above indicate that *EIN3* and *EIL1* are involved in basal resistance to *P. syringae* bacteria, which suggests a role in PTI. Perception of flg22 and other PAMPs activates *PR1* gene expression and callose deposition at the cell

wall. Indeed, the *eil1-1* mutant and *ein3-1 eil1-1* double mutant showed constitutive *PR1* expression, and the *ein3-1* mutant showed elevated *PR1* expression when inoculated with the DC3000 (Figure 3A) and *hrpL* mutant bacteria (Figure 3B). The *hrpL* mutant lacks the HrpL transcription factor required for expression of type III genes required for effector secretion and is thus thought to be a nonpathogenic bacterium carrying a collection of PAMPs. The elevated *PR1* expression in *ein3-1* plants in response to *hrpL* mutant bacteria suggests that EIN3 plays a role in PTI responses. To further determine if *EIN3* and *EIL1* play a role in PTI defenses, we treated the *ein3-4* and *ein3-1 eil1-1* mutants with flg22 and quantitatively examined callose deposition. Figure 3C shows that flg22 induced greater callose deposition in the *ein3-1 eil1-1* double mutant, whereas the *ein3-4* mutant had significantly less callose. Similarly, when treated with the DC3000 *hrpL* mutant bacteria, greater callose deposition was observed in the *ein3-1 eil1-1* double mutant, and reduced callose depositon was seen in the *ein3-4* mutant (Figure 3C). Bacterial growth assay showed that the *ein3-1 eil1-1* double mutant was slightly more resistant, whereas the *ein3-4* mutant was slightly more susceptible to the *hrpL* mutant bacteria compared with Col-0 (Figure 3D). Together, these results indicate that EIN3 and EIL1 are negative regulators of the PTI defenses.

EIN3 and EIL1 Globally Repress PAMP Response Genes

To better understand how EIN3 and EIL1 repress PTI defenses, we conducted microarray analysis on the *ein3-1 eil1-1* double

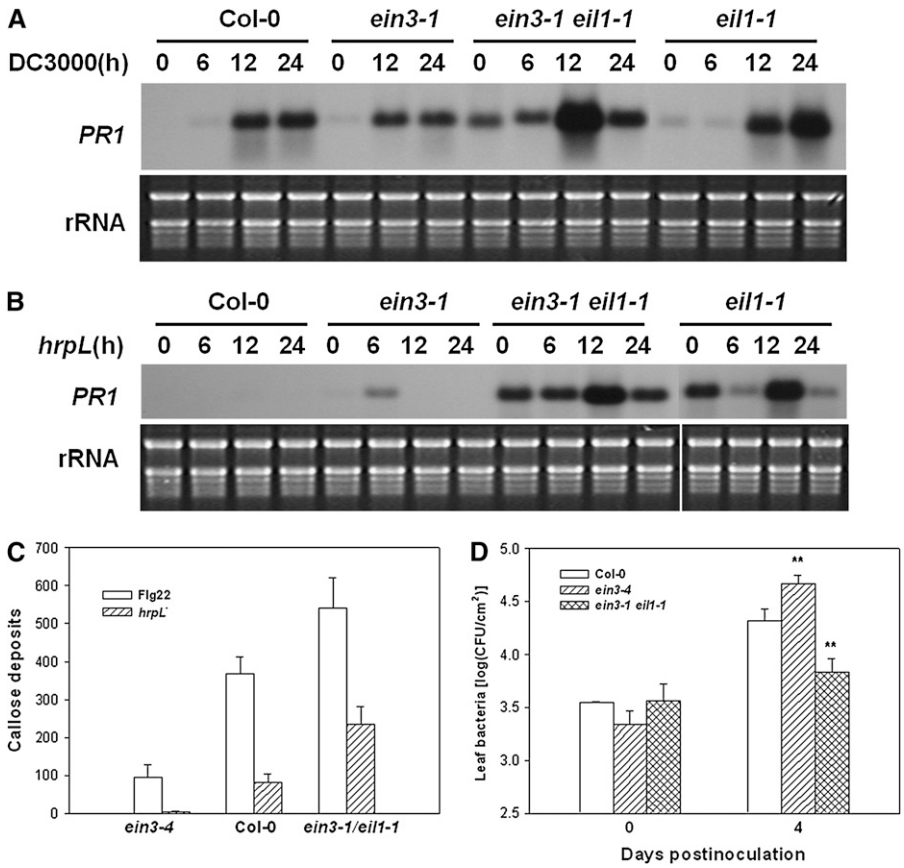

Figure 3. *EIN3* and *EIL1* Negatively Regulate PTI Responses.

(A) RNA gel blot analysis of *PR1* expression in response to DC3000 bacteria. Plants of the indicated genotypes were inoculated with DC3000 bacteria, and RNA was isolated at the indicated times for RNA gel blot analysis.

(B) *PR1* expression in response to the *hrpL* mutant bacteria. Longer exposure was used because the *hrpL* mutant bacteria induce weaker *PR1* expression. Plants of the indicated genotypes were inoculated with *hrpL* mutant bacteria, and RNA was isolated at the indicated times for RNA gel blot analysis.

(C) Callose deposition of wild-type, *ein3-4*, and *ein3-1 eil1-1* mutant plants in response to flg22 or *hrpL* mutant bacteria. Leaves were treated with flg22 or *hrpL* mutant bacteria, stained with aniline blue, and callose deposits/0.1 mm^2 were quantified under a fluorescence microscope. The result is a representative of three experiments.

(D) Bacterial growth assay on plants inoculated with the *hrpL* mutant bacteria. * and ** indicate significant difference at 0.05 and 0.01, respectively, between mutants and Col-0 at the same time point (Student's *t* test). The result is a representative of three experiments.

For **(C)** and **(D)**, each data point consisted of at least three samples. Error bars indicate SD.

mutant using Affimetrix ATH1 oligonucleotide microarray. The *ein3-1 eil1-1* double mutant and Col-0 plants were treated with water or flg22 for 3 h, and three biological replicates were examined. As reported previously, a large number of genes were induced or repressed by the flg22 treatment in the Col-0 plants (Zipfel et al., 2004; Navarro et al., 2004). Using a q value < 0.05 and a fold change of >2 as a cutoff, 1467 genes were identified as flg22 induced and 1819 genes as repressed by flg22 in Col-0 plants (see Supplemental Data Sets 1 and 2 online). A total of 713 genes were upregulated and 335 genes were downregulated in water-treated *ein3-1 eil1-1* plants compared with Col-0 (Figure 4). Nine genes upregulated in the *ein3-1 eil1-1* double mutant were randomly selected and verified by quantitative real-time RT-PCR (see Supplemental Table 2 online). The difference between *ein3-1 eil1-1* and Col-0 plants diminished following flg22 treatment. A total of 459 of the 713 upregulated genes are flg22-induced genes (Figure 4B; see Supplemental Data Set 3 online). The overlap is highly significant (P value $1.69e^{-317}$). Conversely, 226 of the 335 downregulated genes are normally repressed by flg22 treatment (P value $4.84e^{-165}$; see Supplemental Data Set 4 online). These results indicate that EIN3 and EIL1 primarily repress PAMP-triggered transcription programming.

EIN3 and EIL1 Negatively Regulate the Transcription of *SID2* and Repress SA Accumulation

We also compared the genes upregulated in the *ein3-1 eil1-1* double mutant with genes induced by SA. An analysis of data from the AtGenExpress consortium (Shimada et al., submission number: ME00364) identified 321 SA-induced genes at 3 h (q value ≤ 0.05 and fold change ≥2; see Supplemental Data Set 5 online). A total of 96 of these genes are upregulated in the *ein3-1 eil1-1* mutant plants (P value $3.0e^{-64}$) (see Supplemental Data Set 3 online). These include *EDS1*, *PAD4*, *NPR1/NIM1-interacting1* (*NIMIN1*), *PBS3*, and *SAG101*, which are known to mediate SA-dependent defenses (Loake and Grant, 2007). A close examination showed that genes encoding chorismate synthase and isochorismate synthase 1 (ICS1/SID2) were highly elevated in the *ein3-1 eil1-1* double mutant. These two enzymes work in concert downstream of the Shikimate pathway leading to the biosynthesis of isochorismate, which likely is a major precursor for SA biosynthesis (Wildermuth et al., 2001). Indeed, *SID2* is known to be required for SA accumulation during pathogen infection (Wildermuth et al., 2001). Consistent with the increased expression of SA pathway genes in microarray results, quantitative real-time RT-PCR showed increased expression of *PR1*, *PR2*, and *SID2* in *ein3-1 eil1-1* double mutant plants (Figure 5A). We therefore tested if EIN3 and EIL1 are required for transcriptional repression of *SID2* and the chorismate synthase gene. Promoters of these genes were fused to the firefly luciferase (*LUC*) reporter gene and transfected into protoplasts prepared from Col-0, *ein3-1*, and *ein3-1 eil1-1* double mutant plants. Consistent with a role in repressing *SID2* and the chorismate synthase gene promoters, both reporter genes showed increased expression in the *ein3-1 eil1-1* double mutant protoplasts, which was 30- to 100-fold greater than that in the Col-0 protoplasts

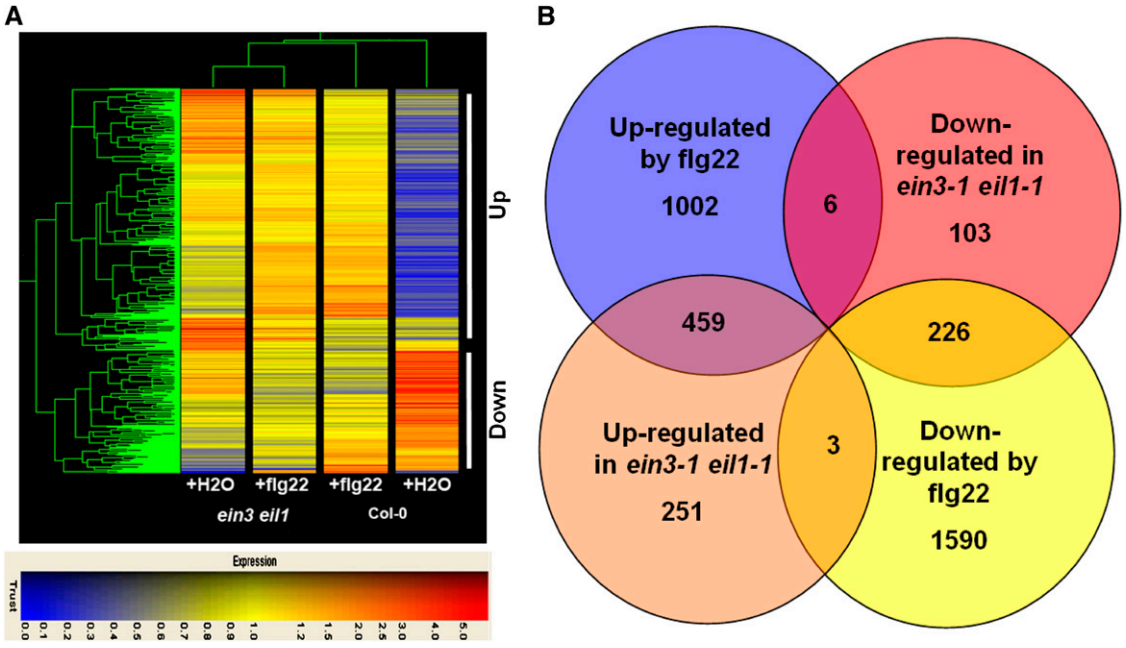

Figure 4. Microarray Analysis of Genes Regulated by EIN3/EIL1 and flg22.

(A) Hierarchical clustering of genes differentially regulated in Col-0 and *ein3-1 eil1-1* plants. Clustering was performed using uncentered Pearson correlation and complete linkage clustering and was visualized with TREEVIEW (Eisen et al., 1998). The 1048 probe sets differentially expressed in water-treated Col-0 and *ein3-1 eil1-1* plants were used for the analysis. Genes upregulated and downregulated in water-treated *ein3-1 eil1-1* plants are indicated. Colors indicate normalized hybridization signal on a scale of 0 to 5.

(B) Venn diagram of genes commonly regulated by EIN3/EIL1 and flg22.

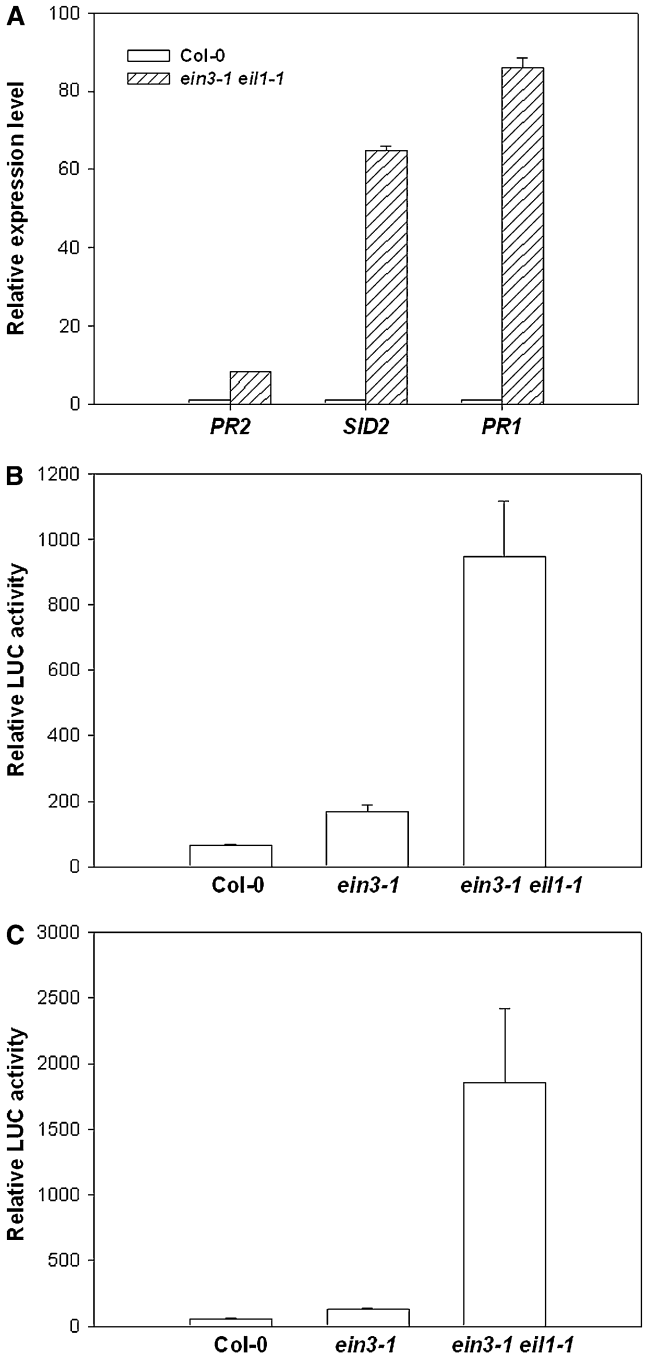

Figure 5. Elevated Expression of SA Pathway Genes in *ein3-1 eil1-1* Double Mutant Plants.

(A) Quantitative real-time RT PCR analysis of *PR1*, *PR2*, and *SID2* gene expression. Expression level for each gene was normalized to that of the wild type (Col-0).

(B) Chorismate synthase promoter-LUC activity in protoplasts isolated from the wild type and *ein3-1 eil1-1* double mutant. Relative LUC activity represents arbitrary luminescence units.

(C) *SID2* promoter-LUC activity in protoplasts isolated from Col-0 and the *ein3-1 eil1-1* double mutant.

Each data point consisted of at least three replicates. Error bars indicate SD. The experiments were performed twice with similar results.

(Figures 5B and 5C). We also tested if the *ein3-4* mutation caused a stronger repression of the *SID2* promoter. Because the *ein3-4* mutant contained the *RAP2.6-LUC* reporter gene, we transfected a *SID2* promoter-β-glucuronidase (*GUS*) construct into protoplasts derived from *ein3-4* and wild-type *RAP2.6-LUC* transgenic plants. The *SID2-GUS* reporter activity in *ein3-4* was ~10% of that in the wild type (see Supplemental Figure 3 online).

We determined if the increased expression of chorismate synthase and *SID2* genes was correlated to SA accumulation in the *ein3-1 eil1-1* plants. Much higher levels of total SA were observed in the *ein3-1 eil1-1* plants, particularly when uninoculated plants were compared (Figure 6A; see Supplemental Figure 4 online). Inoculation of the *ein3-1 eil1-1* double mutant with DC3000 bacteria only modestly elevated the amount of total SA. The *ein3-1 eil1-1* plants also contained slightly more free SA compared with Col-0 (Figure 6B). Thus, EIN3 and EIL1 repress *SID2* and negatively regulate SA biosynthesis.

EIN3 Directly Targets the *SID2* Promoter

We next tested if EIN3 directly targets the *SID2* promoter using chromatin immunoprecipitation (ChIP). Because the EIN3 protein exists at an extremely low level, attempts to test endogenous EIN3-*SID2* promoter binding were unsuccessful. We therefore constructed transgenic plants expressing EIN3-3×FLAG fusion protein under an estradiol-inducible promoter (Zuo et al., 2000). Chromatin immunoprecipitated with the anti-FLAG antibody was profoundly enriched in fragment P5 (located −120 to −324 bp upstream of the transcription start site; Figure 7). The EIN3/EIL family protein was reported to bind DNA with a consensus sequence of A(C/T)G(A/T)A(C/T)CT (Kosugi and Ohashi, 2000; Yamasaki et al., 2005). However, only positions 1, 3, and 5 of this sequence were experimentally demonstrated to be important for recognition. P5 contains three relaxed EIN3 binding sites A(C/T)G(A/T)A(C/T). DNA fragments further upstream showed detectable but less enrichment with EIN3-3×FLAG. By contrast, a fragment from the second intron of *SID2* and an actin promoter sequence did not show detectable enrichment by EIN3-3×FLAG (Figure 7). These results indicate a specific association of EIN3-3×FLAG with the *SID2* promoter sequence, particularly fragment P5.

DNA electrophoresis mobility-shift assay (EMSA) was performed to determine if EIN3 directly binds the *SID2* promoter. A truncated EIN3 protein (amino acids 141 to 352) containing the DNA binding domain was expressed as a glutathione *S*-transferase (GST) fusion protein in *Escherichia coli* and affinity purified. The GST-EIN3 protein was capable of binding to the radiolabeled P5 DNA fragment in vitro (Figure 7C). The addition of unlabeled P5 DNA fragment blocked the binding. Furthermore, the oligonucleotide obs1 containing the core EIN3 binding sequence (ATGTAC; Kosugi and Ohashi, 2000) was similarly

capable of competing with the binding. By contrast, the oligo nucleotides obsm2 and obsm123, in which the core sequence was respectively changed to ATTTAC and CTTTCC, were unable to compete with P5-EIN3 binding. Together, these results demonstrate that EIN3 is capable of binding the conserved EIN3 binding element in the *SID2* promoter both in vivo and in vitro.

To determine the importance of EIN3 binding sequence in the *SID2* promoter, the P5 sequence was removed from the *SID2-GUS* construct and transfected into *ein3-1 eil1-1* and Col-0 protoplasts. The *SID2P5Δ-GUS* reporter activity was greatly increased in Col-0 protoplasts but not in *ein3-1 eil1-1* protoplasts (see Supplemental Figure 5 online), indicating that the P5 sequence contributes to transcriptional repression of *SID2* promoter by EIN3 and EIL1.

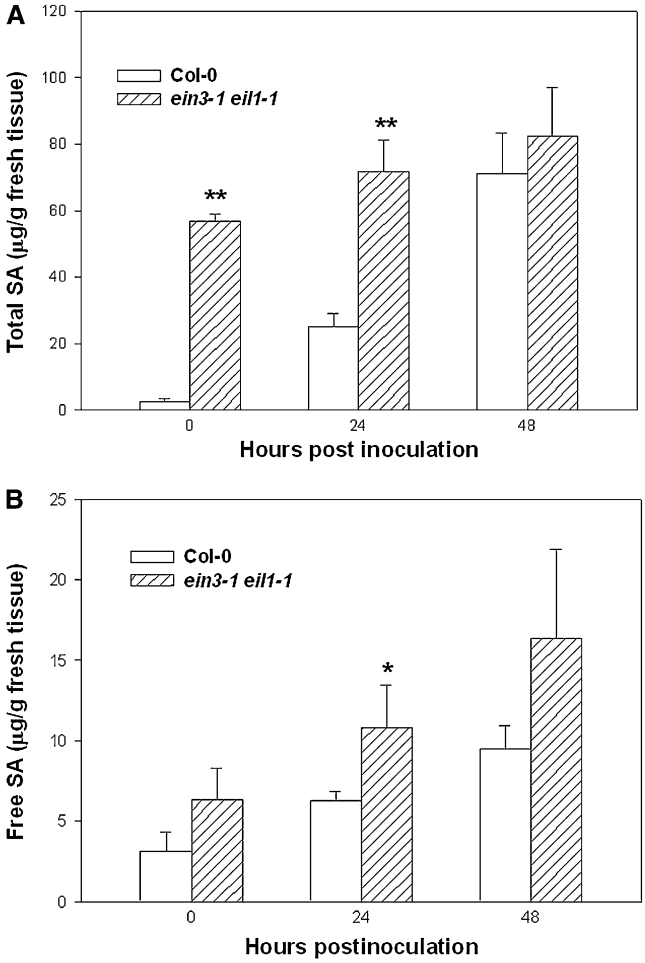

Figure 6. Heightened SA Level in *ein3-1 eil1-1* Double Mutant Plants.

(A) Total SA in the *ein3-1 eil1-1* and Col-0 plants.
(B) Free SA in the *ein3-1 eil1-1* and Col-0 plants.
Plants were inoculated with DC3000 bacteria (10^6 cfu/mL) for the indicated hours, and tissues were collected for SA extraction. Each data point consisted of three replicates. Error bars indicate SD. * and ** indicate significant difference at 0.05 and 0.01, respectively (Student's *t* test). The experiment was repeated twice with similar results.

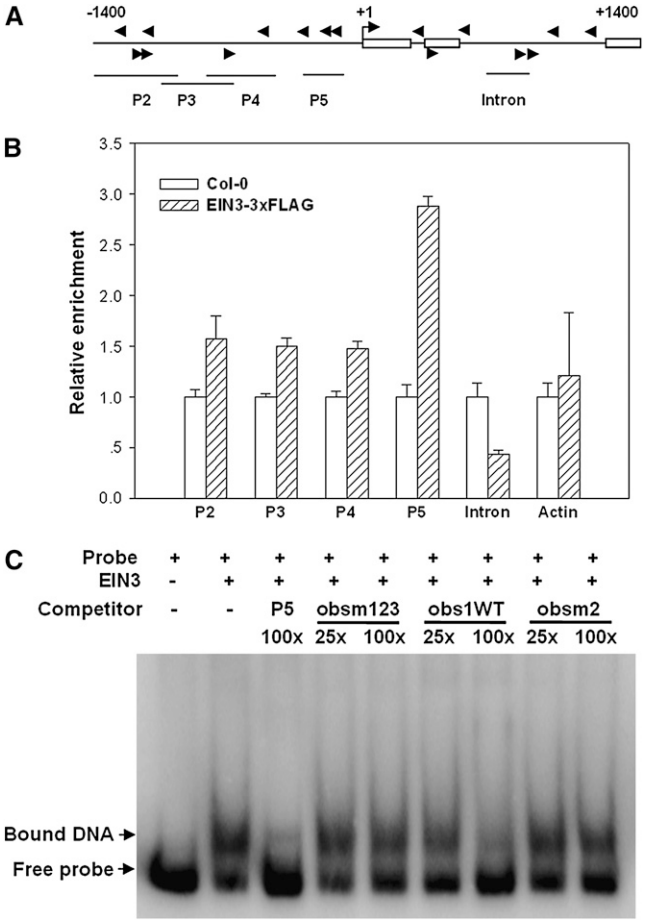

Figure 7. EIN3 Binds the *SID2* Promoter.

(A) Schematic diagram of potential EIN3 binding sites (arrows) and DNA fragments used for ChIP experiments. Shown are 1.4-kb upstream sequence and part of the coding sequence for *SID2*. Boxes are exons, and the translational start site (ATG) is shown at position +1.
(B) Enrichment of the indicated DNA fragments following ChIP using anti-FLAG antibody. Chromatin from wild-type and transgenic plants expressing EIN3-3×FLAG was immunoprecipitated with an anti-FLAG antibody, and the presence of the indicated DNA in the immune complex was determined by quantitative real-time PCR. The amounts of DNA amplified from the EIN3-3×FLAG seedlings were normalized to that from Col-0 plants. The Actin promoter fragment was used as a negative control. The experiment was repeated three times with similar results.
(C) EMSA assay for EIN3-P5 DNA fragment binding in vitro. Radiolabeled P5 DNA fragment was incubated with GST-EIN3 protein, and the free and bound DNA (arrows) were separated in an acrylamide gel. Where indicated, the P5 fragment and oligonucleotide primers WTobs1 (5′-AACGATGTACCTGGTCGTATT-3′), obsm2 (5′-GTACATTTACCTGGACCGTGA-3′), and obsm123 (5′-GTACCTTTCCCTGGACCGTGA-3′) were used as competitor DNA.

SID2 Is Responsible for Enhanced Defenses in the *ein3-1 eil1-1* Double Mutant

The results described above suggest that EIN3 and EIL1 repress PTI defenses through repressing *SID2*. We therefore introduced the *sid2-2* mutation into the *ein3-1 eil1-1* double mutant. The

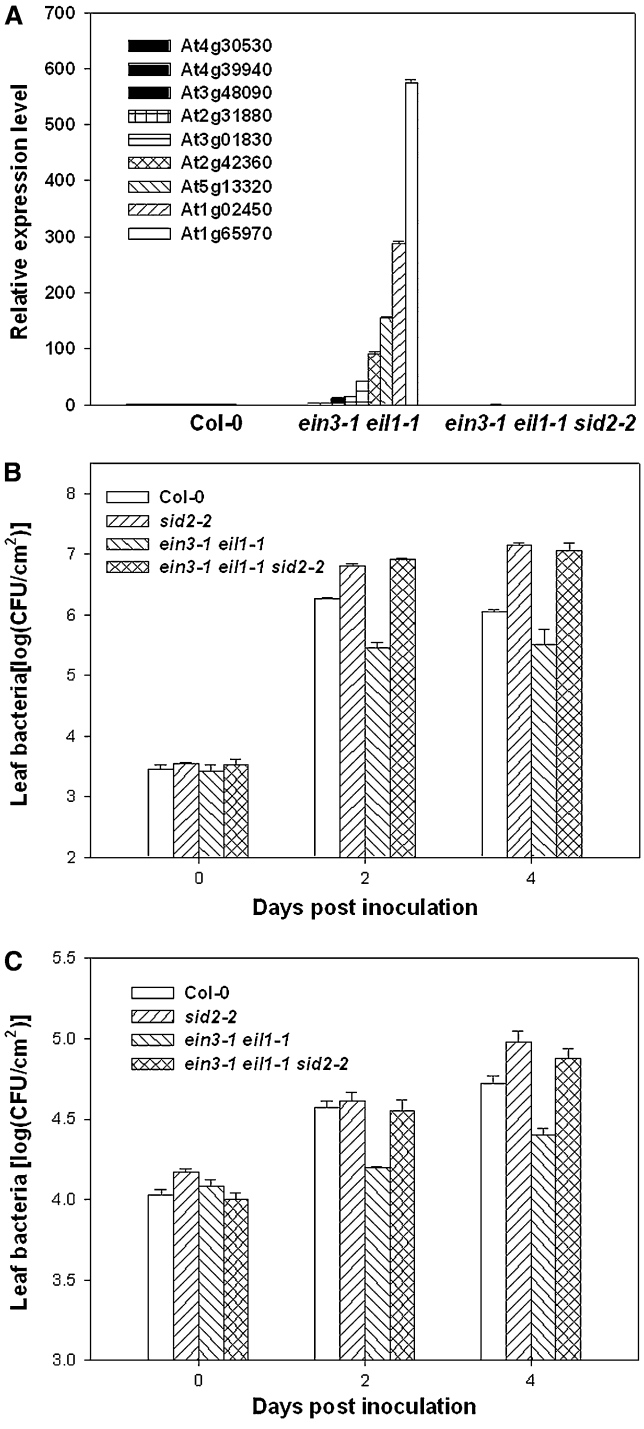

Figure 8. *SID2* Is Required for Heightened Defenses and Enhanced Disease Resistance in *ein3-1 eil1-1* Plants.

(A) *SID2* is required for enhanced expression of flg22 response genes in the *ein3-1 eil1-1* double mutant. Expression levels of the indicated genes in untreated wild-type (Col-0), *ein3-1 eil1-1* double, and *ein3-1 eil1-1 sid2-2* triple mutant plants were determined by quantitative RT-PCR. The value shows normalized expression level for each gene. The experiment was repeated three times with similar results.

expression of the nine selected genes upregulated in the *ein3-1 eil1-1* double mutant was largely restored to wild-type levels in the *ein3-1 eil1-1 sid2-2* triple mutant (Figure 8A), indicating that *SID2* and possibly other SA biosynthetic genes were responsible for the elevated defense gene expression. To further test if the enhanced *SID2* expression contributes to the enhanced disease resistance in the *ein3-1 eil1-1* double mutant, we inoculated the *ein3-1 eil1-1 sid2-2* triple mutant with DC3000 and the *hrpL* mutant bacteria. Bacterial growth assays indicated that the triple mutant was completely susceptible to the bacteria compared with the *sid2-2* mutant (Figures 8B and 8C), indicating that the *SID2*-mediated resistance accounted for the elevated basal resistance in the *ein3-1 eil1-1* double mutant.

ein2 and *ebf* Mutants Display Altered Defenses to *P. syringae*

The negative regulation of SA biosynthesis by EIN3 and EIL1 raises a possibility that the ET pathway may negatively regulate the SA pathway through these two transcription factors. We therefore tested if other ET pathway mutants also affected *P. syringae* bacterial resistance. EBF1 and EBF2, two closely related F-box proteins, direct the ubiquitination and subsequent degradation of EIN3 and EIL1. The *ebf1 ebf2* double mutants overaccumulate EIN3 and EIL1 (Guo and Ecker, 2003; Potuschak et al., 2003). We tested if the *ebf1-1 ebf2-1* double mutant (Potuschak et al., 2003) was more susceptible to DC3000 bacteria. Indeed, the *ebf1-1 ebf2-1* double mutant supported ~20-fold more bacterial growth than did the wild type (Figure 9A). Another important ET pathway component, EIN2, is an integral membrane protein that acts upstream of EIN3 and EIL1 to regulate the ET pathway (Guo and Ecker, 2004). In contrast with the *ebf1-1 ebf2-1* mutant, the *ein2-1* mutant is unable to accumulate EIN3 (Guo and Ecker, 2003). In an early report, it was shown that the *ein2-5* mutant exhibited reduced disease symptoms when inoculated with virulent *P. syringae* bacteria (Bent et al., 1992). However, whether this mutant supports less bacterial growth has not been carefully examined. Our repeated bacterial growth assays showed that *ein2-1* plants supported 10-fold less DC3000 bacterial growth than did Col-0 plants (Figure 9B). We also examined *PR1* expression in the *ein2-1* mutant plants. Consistent with a previous report (Lawton et al., 1994), *ein2-1* plants showed constitutive *PR1* expression (Figure 9C). In addition, greater *PR1* expression was observed

(B) *SID2* is required for enhanced resistance to DC3000 in the *ein3-1 eil1-1* plants. Wild-type (Col-0), *sid2-2*, *ein3-1 eil1-1* double, and *ein3-1 eil1-1 sid2-2* triple mutant plants were inoculated with DC3000 bacteria, and bacterial population in the leaf was determined at the indicated times. The experiment was repeated three times with similar results.

(C) *SID2* is required for enhanced resistance to the *hrpL* mutant bacteria in *ein3-1 eil1-1* plants. The experiment was repeated twice with similar results. Wild-type (Col-0), *sid2-2*, *ein3-1 eil1-1* double, and *ein3-1 eil1-1 sid2-2* triple mutant plants were inoculated with *hrpL* mutant bacteria, and bacterial population in the leaf was determined at the indicated times.

Each data point consisted of at least three replicates. Error bars indicate SD.

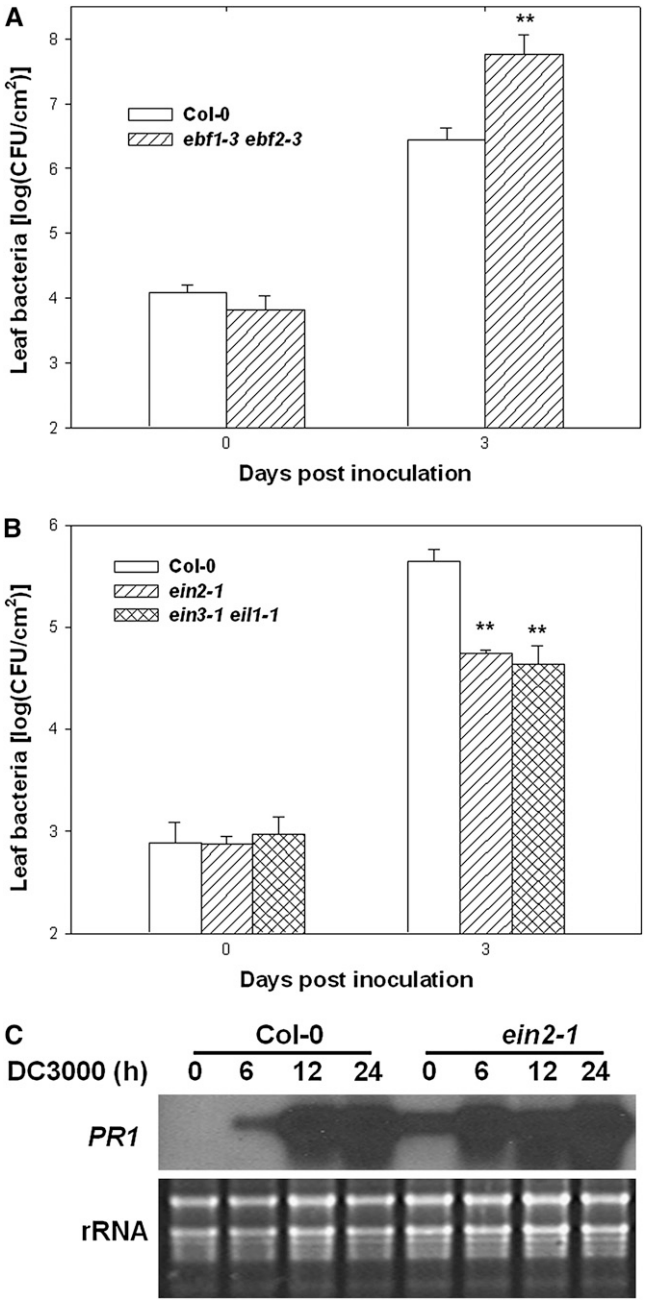

Figure 9. *ein2* and *ebf* Mutants Display Altered Defenses to *P. syringae* DC3000.

(A) Enhanced susceptibility in *ebf1-3 ebf2-3* double mutants. Wild-type (Col-0) and *ebf1-3 ebf2-3* plants were inoculated with DC3000 bacteria, and bacterial population in the leaf was determined at the indicated times.

(B) Enhanced disease resistance in *ein2-1*. Wild-type (Col-0) and *ein2-1* plants were inoculated with DC3000 bacteria, and bacterial population in the leaf was determined at the indicated times.

(C) Enhanced *PR1* expression in *ein2-1* mutant plants in response to DC3000. Plants of the indicated genotypes were inoculated with DC3000 for the indicated times, and RNA was isolated for RNA gel blot analysis. Each data point consisted of three replicates. Error bars indicate SD.

in *ein2-1* plants 6 h after inoculation with DC3000 bacteria (Figure 9C). Together, these results support that EIN3 and EIL1 mediate a crosstalk between the ET pathway and the SA pathway.

DISCUSSION

A major challenge in examining the signal transduction network in PTI defenses is to identify novel regulators in this network. In this study, we demonstrate that EIN3 and EIL1 negatively regulate PTI resistance. The *ein3-1 eil1-1* double mutant showed elevated callose deposition and *PR1* expression when treated with flg22 or *hrpL* mutant bacteria. Moreover, plants lacking both EIN3 and EIL1 display enhanced resistance to compatible, incompatible, and nonpathogenic *P. syringae* strains. By contrast, plants that overaccumulate EIN3 protein (*35S-EIN3* transgenic plants and *ebf1-3 ebf2-3* double mutants) are compromised in defenses and show enhanced disease susceptibility. Furthermore, microarray analysis uncovered that the majority of genes upregulated in the *ein3-1 eil1-1* double mutant are PAMP response genes in wild-type plants. These results demonstrate that EIN3 and EIL1 negatively regulate disease resistance by repressing the PAMP-triggered transcriptional program. The flg22-induced accumulation of EIN3 protein suggests a negative feedback loop in the regulation of PTI defenses.

Microarray analysis showed a strong association of genes repressed by EIN3/EIL1 and genes induced by SA. Among the genes highly elevated in the *ein3-1 eil1-1* double mutant are *EDS1* and *PAD4*, which are known to amplify the SA defenses through a positive feedback loop (Feys et al., 2001). The SA biosynthetic gene *SID2* was also strongly expressed in the *ein3-1 eil1-1* double mutant. Reporter assays showed that the promoter of *SID2* is hyperactive in *ein3-1 eil1-1* double mutant protoplasts, suggesting that EIN3 and EIL1 transcriptionally target *SID2* to regulate defenses. ChIP and EMSA experiments showed that the EIN3 protein can specifically bind to the promoter of *SID2* in plants and in vitro. Furthermore, elimination of the major EIN3 binding sequence P5 resulted in a promoter less responsive to repression by EIN3. Taken together, these results support that EIN3, and possibly EIL1, directly targets the *SID2* promoter to repress plant disease resistance. Importantly, the *sid2* mutation completely eliminates the enhanced disease resistance phenotype caused by the *ein3-1* and *eil1-1* mutations. Moreover, the nine selected genes constitutively expressed at a high level in the *ein3-1 eil1-1* double mutant were all reduced to normal levels by the *sid2-2* mutation. These results collectively demonstrate that *SID2* is a key target gene under transcriptional control of EIN3 and EIL1. Thus, EIN3 and EIL1 negatively regulate PTI defenses primarily by downregulating *SID2* transcription. It was recently shown that PAMPs can induce SA biosynthesis in a *SID2*-dependent manner (Tsuda et al., 2008) and that the SA signaling pathway is required for the full activation of PTI defenses (Mishina and Zeier, 2007; Tsuda et al., 2008). Thus, *SID2* is

* and ** indicate significant difference at 0.05 and 0.01, respectively, between mutants and Col-0 at the same time point (Student's *t* test). The experiments were repeated twice with similar results.

subjected to both positive and negative regulation during PTI defenses.

EIN3 and EIL1 have been shown to activate transcription of ET response genes, such as *ERF1* (Solano et al., 1998). The defense suppression function of EIN3, however, is uncoupled from ET-induced plant development. The *ein3-4* allele, which carries a P216S substitution, enhances EIN3 protein stability in plants through an unknown mechanism. Similar to the *ein3-1* null mutant, *ein3-4* is a recessive mutant defective in ET-induced triple response, indicating that P216 is critical for ET-induced plant development. Contrary to *ein3-1*, the *ein3-4* mutant phenocopies EIN3 overexpression plants and represses disease resistance to *P. syringae* bacteria. Furthermore, *ein3-4* exhibits greater repressor activity toward the *SID2* promoter and acts as a semidominant allele to suppress disease resistance to *P. syringae* (*avrB*), indicating that the EIN3^{P216S} mutant protein is fully capable of repressing plant defenses. It is clear that the defense suppression activity of EIN3 is independent of ET-induced plant development. Thus, EIN3 possesses both transcriptional activator and repressor activities, depending on the target genes. It has long been recognized that some transcription factors can act as both activator and repressor (Roy et al., 1998). It is conceivable that EIN3 and EIL1 may interact with other transcription factors or cofactors to repress *SID2* transcription. For example, WRKY54 and WRKY70 are known to repress *SID2* transcription, although it is not clear if they directly bind to the *SID2* promoter (Wang et al., 2006).

Plant immunity is actively repressed in the absence of pathogen attack, and unregulated defenses are detrimental to plant growth and development. Thus, negative regulation is an integral part of plant immunity regulatory network (Schwessinger and Zipfel, 2008). An important feature of this network is crosstalk among different hormonal pathways (Spoel and Dong, 2008). The crosstalk allows plants to devote resources for optimum growth in the absence of pathogen attack and choose appropriate defense strategies when attacked by pathogens of different lifestyles. For example, JA and ET play an important role in plant development and mediate defenses primarily against necrotrophs and insects, whereas SA mediates resistance against biotrophic pathogens (Spoel and Dong, 2008). The crosstalk between JA and SA pathways is primarily antagonistic and has been studied extensively; here we show direct crosstalk between the ET and SA pathways. Consistent with this, elevated *PR1* expression in an *ein2* mutant has been observed in a previous report (Lawton et al., 1994). Our findings that ET signal transduction pathway components EIN2, EBF1, EBF2, EIN3, and EIL1 regulate disease resistance show crosstalk between the ET and SA signaling pathways in which EIN3 and EIL1 act as a regulatory node to fine-tune PTI defenses in plants.

METHODS

Plant Materials and Mutant Screen

Plant materials used in this study include a *RAP2.6-LUC* line (He et al., 2004), wild-type Col-0, *ein3-1* (Chao et al., 1997), *eil1-1* (Alonso et al., 2003), *ein3-1 eil1-1* (Alonso et al., 2003), *ebf1-1 ebf2-1* (Potuschak et al., 2003), *sid2-2* (Dewdney et al., 2000), *ein2-1* (Roman et al., 1995), and 35S:*EIN3* transgenic line (Chao et al., 1997). Plants were grown in a growth room with 75% humidity under 12-h daylight at 20°C (night) and 23°C (day). The *RAP2.6-LUC* mutant population, mutant screening, and *RAP2.6-LUC* reporter assay are as described (Shang et al., 2006).

For complementation test, the *rrb6* mutant was crossed to *ein3-1*, and F1 seedlings were scored for triple response in the presence of 10 μM ACC in Murashige and Skoog medium according to Guo and Ecker (2003). The *ein3-1 eil1-1* double mutant was crossed to *sid2-2* to generate the *ein3-1 eil1-1 sid2* triple mutant. The triple mutant was confirmed by PCR. The single nucleotide substitution in *ein3-1* and was verified using a cleaved-amplified polymorphic sequence marker (primers 5′-TACCAAGTATCAAGCGGAG-3′ and 5′-AGGCCACCAATCCTCTTTC-3′; *Hae*III digest). *eil1-1* contains a transposon insertion and was verified by PCR using primers 5′-GGGAATGGTGGAAAGATAAG-3′ and 5′-CTTTCGCCGTCATCTTATCC-3′. *sid2-2* carries an ~50-bp deletion in exon IX and was verified using PCR primers 5′-TTCTTCATGCAGGGGAGGAG-3′, 5′-CAACCACCTGGTGCACCAGC-3′, and 5′-AAGCAAAATGTTTGAGTCAGCA-3′ (Wildermuth et al., 2001).

To construct transgenic plants expressing EIN3-3FLAG, *EIN3* cDNA was PCR amplified from reverse transcription product with primers 5′-AGGTTCGAAGAACCATATGGATACATCTTG-3′ and 5′-AAACTCGAGATGATGTTTAATGAGATGGGAATG-3′, digested with *Xho*I and *Csp*45I, inserted into a pER8-derived plasmid containing triple FLAG tag (Zuo et al., 2000; Li et al., 2005), introduced into *Agrobacterium tumefaciens* strain GV3101, and transformed into *Arabidopsis thaliana* Col-0 (Clough and Bent, 1998). A stable transgenic line expressing EIN3-3FLAG was selected and used in the ChIP experiment.

Bacterial Growth Assay

Bacterial strains used include *Pseudomonas syringae* pv *tomato* DC3000 and the *hrpL* mutant strain derived from DC3000 (Zwiesler-Vollick et al., 2002). Bacteria were infiltrated into 5-week-old *Arabidopsis* plants at 10^5 colony-forming units (cfu)/mL or 10^6 cfu/mL for bacterial growth assay as described (He et al., 2004).

RNA Gel Blot and Quantitative Real-Time RT-PCR Analyses of Gene Expression

Plants were infiltrated with 10^6 cfu/mL bacteria or water, and leaves were collected at the indicated times for RNA isolation. Ten micrograms of total RNA was loaded in each lane, and the RNA gel blot was hybridized with the indicated radiolabeled probes. For quantitative real-time RT-PCR, RNA was reverse transcribed into cDNA using SuperScript reverse transcriptase (Invitrogen). Real-time PCR was then performed using SYBR Green Mix and specific primers listed in Supplemental Table 3 online. The expression level was normalized to actin control.

Microarray Analysis

Wild-type and *ein3-1 eil1-1* plants were treated with 2 μM flg22 or water for 3 h prior to RNA isolation. Each treatment contains three biological replicates for each genotype (each replicate consisting of a pool of RNA from six plants) for a total of 12 array hybridizations. Affymetrix ATH1 arrays were used for hybridization. Experiment data from the AtGen Express consortium were used for analyzing SA-regulated genes (Shimada et al., submission number: ME00364). GCOS software and MAS5 algorithm were used for data collection and normalization. To analyze the differentially expressed genes between control and treatment, fold change and significance analysis of microarrays (two class-paired; Tusher et al., 2001) were applied. A q value ≤ 0.05 and fold change ≥ 2 between treatment and control samples were considered as cutoff,

and the induced or repressed genes were selected. Contingency tests were conducted for random overlap between two gene lists, and P value was calculated using GeneSpring GX software (http://www.chem.agilent.com/en-US/Support/FAQs/Informatics/GeneSpring%20GX/Lists2/Pages/KB001066.aspx).

Callose Staining

Five-week-old *Arabidopsis* leaves were infiltrated with 2 μM flg22 or 10^6 cfu/mL *hrpL* mutant bacteria for 14 h, cleared, stained with aniline blue (Hauck et al., 2003), and mounted in 50% glycerol, and epifluorescence was visualized with a fluorescence microscope under UV light. The number of callose deposits per microscopic field of 0.1 mm^2 was calculated from six leaves using the Image J software (http://www.uhnresearch.ca/wcif).

SA Quantitation

Total and free SA was quantitated according to Li et al. (1999). Briefly, frozen leaf tissue (0.1 g) was ground in liquid nitrogen, extracted twice with 90% methanol by vortex and sonication, and the sample (free SA) was dried under vacuum. The free SA sample was treated with β-glucosidase to yield total SA sample. Both free SA and total SA, samples were extracted once with 5% trichloroacetic acid and three times with 100/99/1 (vol) ethylacetate/cyclopentane/isopropanol. The dried SA was then resuspended in 250 μL mobile phase (0.2 M KAc and 0.5 mM EDTA, pH 5) and separated through a 100 × 4.6 sperisorb DDS2 column (Keystone Scientific) with a particle size of 3 μm and a pore size of 80 A° at a mobile-phase flow rate of 1 mL/min. Fluorescent detection was performed on an HPLC spectrofluorescence detector with a Xenon-mercury arc lamp at an excitation/emission wavelength of 295/405 nm.

Immunoblot Assay

Total protein was extracted from 4-d-old etiolated seedlings. EIN3 protein was determined by immunoblot using antibodies raised against recombinant EIN3 protein (Guo and Ecker, 2003). For loading control, the blot was hybridized to anti-HSP90 antibodies (Provided by Gang Zhi, Antibody Core Facility, National Institute of Biological Sciences).

ChIP

ChIP was performed as described previously with minor modifications (Gendrel et al., 2005). Briefly, wild-type and EIN3-3FLAG transgenic seeds were sterilized and grown on half Murashige and Skoog medium in the presence of 10 μM β-estrodial under continuous white light for 8 d. Seedlings (2.5 g) were fixed in 1% formaldehyde for 15 min in vacuum and neutralized with 0.125 M glycine in vacuum for additional 5 min. After washing twice with cold, sterilized water, the tissue was ground in liquid nitrogen. Nuclei were isolated and sonicated. Sonicated chromatin supernatant (300 μL) was diluted to 3 mL, and 20 μL of protein A-agarose bead (Upstate) was added for preclear at 4°C for 1 h. The chromatin was then divided into two 1.5-mL aliquots. Twenty microliters of mouse anti-FLAG M2-agarose beads (Sigma-Aldrich) were added to one tube, 20 μL of protein A-agarose beads were added to the other as "no antibody control." After incubating at 4°C for overnight, beads were washed with low salt wash buffer, high salt wash buffer, and TE buffer. Elution and reversed cross-linking was done as previously described (Gendrel et al., 2005). Eluates were treated with Proteinase K (10 mg/mL; Sigma-Aldrich) and RNase for 2.5 h at 45°C, phenol/chloroform extracted, and ethanol precipitated with the aid of 20 μg glycogen. The purified DNA was resuspended in 80 μL of water. The enrichment of DNA fragments was determined by quantitative real-time PCR using primers listed in Supplemental Table 3 online.

EMSA

To construct plasmid for the expression of recombinant EIN3 protein (amino acids 141 to 352) in *Escheichia coli*, the correspond DNA fragment was amplified by PCR using primers 5′-ACTGGATCCAAGGTTAGGTTTGATCGT-3′ and 5′-ACTCTCGAGTCAGAAGAATTCATAACTTTT-3′ and inserted into *Bam*HI and *Xho*I of the pGEX-6p-1 vector (Pharmacia). For probe, 4 pM PCR-amplified P5 DNA fragment was ^{32}P-labeled using T4-polynucleotide kinase (New England Biolabs) in the presence of 20 μCi [γ-^{32}P]ATP and purified by Sephadex G-50 column. EMSA was performed as described (Kosugi and Ohashi, 2000). Each binding reaction (19 μL) contained 200 ng recombinant protein, 5 ng labeled DNA probe, 0.1 μg poly[d(I-C)] (Sigma-Aldrich), 20 mM HEPES, pH 7.8, 3 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA, 40 mM KCl, and 10% glycerol. After incubation on ice for 30 min, the mixtures were loaded onto 4.5% polyacrylamide gels (29:1) to separate free and bound DNA. For competition experiments, the following primers were used: Obs1, 5′-AACGATGTACCTGGTCGTATT-3′; Obsm2, 5′-GTACATTTACCTGGACCGTGA-3′; and Obsm123, 5′-GTACCTTTCCCTGGACCGTGA-3′ (Kosugi and Ohashi, 2000).

Construct for Promoter-Reporter Assay

A pUC19-GUS-RBS vector was generated from pUC19-35S-FLAG-RBS plasmid (Li et al., 2005), replacing 3×FLAG sequence with GUS sequence between *Xho*I and *Pst*I. To generate *SID2-GUS* construct, a 1.6-kb *SID2* promoter region was amplified from genomic DNA with primers 5′-CACGAATTCTTCGTAGCATCCACAACAC-3′ and 5′-ACTGGTACCTGCAGAAATTCGTAAAGTG-3′ and inserted between *Eco*RI and *Kpn*I sites of pUC19-GUS vector. The *SID2P5Δ-GUS* construct containing a deletion between −324 and −251 (from translational start site) of the *SID2* promoter was generated by overlap extension PCR using primers 5′-TAGACCAAGTAAATGAAGTAGGATTAGAAG-3′ and 5′-CTTCTAATCCTACTTCATTTACTTGGTCTA-3′.

To generate 35S-rLUC construct, the coding sequence of *Renilla* luciferase fragments was amplified from pSP-luc(+) (Promega) using primers 5′-ATAGGTACCATGGCTTCCAAGGTGTACGAC-3′ and 5′-GTACTGCAGTTACTGCTCGTTCTTCAGCAC-3′ and inserted into *Kpn*I and *Pst*I of pUC19-35S-FLAG-RBS plasmid, resulting in pUC19-35S-rLUC-RBS.

To generate *SID2-LUC*, the 3×FLAG sequence in pUC19-35S-FLAG-RBS plasmid (Li et al., 2005) was first replaced with the coding sequence of firefly luciferase gene *LUC* between *Xho*I and *Pst*I, resulting in pUC19-LUC-RBS. The *SID2* promoter was PCR amplified with primers 5′- CACCAATTGAATTCAACTAACGTCCTAT-3′ and 5′-ACTGGTACCTGCAGAAATTCGTAAAGTG-3′, digested with *Mfe*I and *Kpn*I, and inserted into pUC19-LUC-RBS vector, resulting in SID2-LUC construct.

Protoplasts were transfected with plasmids as described (Li et al., 2005). For GUS assay, protoplasts were transfected with *SID2-GUS* or *SID2P5D-GUS* along with 35S-*rLUC* and incubated for 12 to 14 h at room temperature before harvested for GUS activity assay. GUS activity was assayed with methyl umbelliferyl glucoronide (Sigma-Aldrich), and renilla luciferase activity was assayed using a luciferase assay kit (Promega) following the manufacturer's instruction. The GUS/rLUC ratio was used to determine the promoter activity. The SID2-LUC reporter activity was determined as described (Li et al., 2005).

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: *Actin* (At3g18780), *EIN3* (At3g20770), *EIL1* (At2g27050), *SID2* (At1g74710), *PR1* (At2g14610), *PR2* (At3g57260), *EDS1* (At3g48090), *NIMIN1* (At1g02450), *PBS3* (At5g13320), a putative defense-related protein (At4g30530), adenosine-5′-phosphosulfate-kinase (AKN2;

At4g39940), leucine-rich repeat transmembrane protein (At2g31880), calmodulin-related protein (At3g01830), C3HC4-type RING finger family protein (At2g42360), and thioredoxin-dependent peroxidase 2 (At1g65970).

Supplemental Data

The following materials can be found in the online version of this article.

Supplemental Figure 1. Enhanced Disease Symptoms in *ein3-4* Plants.

Supplemental Figure 2. *ein3-4* Is a Semidominant Mutation That Enhances Disease Susceptibility.

Supplemental Figure 3. *ein3-4* Shows Greater Repression of *SID2* Promoter.

Supplemental Figure 4. Total SA Level in *ein3-1 eil1-1* Double Mutant Plants after Bacterial Infection.

Supplemental Figure 5. *SID2* P5 Sequence Contributes to Transcriptional Repression by EIN3 and EIL1.

Supplemental Table 1. Complementation Test of *rrb6* and *ein3-1.*

Supplemental Table 2. Quantitative Real-Time RT-PCR Analysis of Genes Upregulated in *ein3-1 eil1-1*.

Supplemental Table 3. Primers Used for Quantitative PCR Analyses.

Supplemental Data Set 1. Genes Induced by flg22.

Supplemental Data Set 2. Genes Repressed by flg22

Supplemental Data Set 3. Genes Upregulated in Water-Treated ein3 eil1 Plants.

Supplemental Data Set 4 Genes Downregulated in Water-Treated ein3/eil1 Plants.

Supplemental Data Set 5. Genes Induced by SA.

ACKNOWLEDGMENTS

We thank Gang Zhi for preparing anti-HSP90 antibodies, Frederick Ausubel for the *sid2-2* seeds, Pascal Genschik for *ebf1-3 ebf-2-3* seeds, and Mary Wildermuth for sharing *sid2-2* genotyping method. J.Z. was supported by grants from Chinese Ministry of Science and Technology (2003-AA210080).

Received December 22, 2008; revised August 3, 2009; accepted August 16, 2009; published August 28, 2009.

REFERENCES

Alonso, J.M., Stepanova, A.N., Solano, R., Wisman, E., Ferrari, S., Ausubel, F.M., and Ecker, J.R. (2003). Five components of the ethylene-response pathway identified in a screen for weak ethylene-insensitive mutants in Arabidopsis. Proc. Natl. Acad. Sci. USA **100:** 2992–2997.

Ausubel, F.M. (2005). Are innate immune signaling pathways in plants and animals conserved? Nat. Immunol. **6:** 973–979.

Bent, A.F., Innes, R.W., Ecker, J.R., and Staskawicz, B.J. (1992). Disease development in ethylene-insensitive *Arabidopsis thaliana* infected with virulent and avirulent *Pseudomonas* and *Xanthomonas* pathogens. Mol. Plant Microbe Interact. **5:** 372–378.

Bittel, P., and Robatzek, S. (2007). Microbe-associated molecular patterns (MAMPs) probe plant immunity. Curr. Opin. Plant Biol. **10:** 335–341.

Chao, Q., Rothenberg, M., Solano, R., Roman, G., Terzaghi, W., and Ecker, J.R. (1997). Activation of the ethylene gas response pathway in Arabidopsis by the nuclear protein ETHYLENE-INSENSITIVE3 and related proteins. Cell **89:** 1133–1144.

Chisholm, S.T., Coaker, G., Day, B., and Staskawicz, B.J. (2006). Host-microbe interactions: Shaping the evolution of the plant immune response. Cell **124:** 803–814.

Clough, S.J., and Bent, A.F. (1998). Floral dip: A simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana.* Plant J. **16:** 735–743.

Dewdney, J., Reuber, T.L., Wildermuth, M.C., Devoto, A., Cui, J., Stutius, L.M., Drummond, E.P., and Ausubel, F.M. (2000). Three unique mutants of Arabidopsis identify *eds* loci required for limiting growth of a biotrophic fungal pathogen. Plant J. **24:** 205–218.

Durrant, W.E., and Dong, X. (2004). Systemic acquired resistance. Annu. Rev. Phytopathol. **42:** 185–209.

Eisen, M.B., Spellman, P.T., Brown, P.O., and Botstein, D. (1998). Cluster analysis and display of genome-wide expression patterns. Proc. Natl. Acad. Sci. USA **95:** 14863–14868.

Feys, B.J., Moisan, L.J., Newman, M.A., and Parker, J.E. (2001). Direct interaction between the Arabidopsis disease resistance signaling proteins, EDS1 and PAD4. EMBO J. **20:** 5400–5411.

Gendrel, A.V., Lippman, Z., Martienssen, R., and Colot, V. (2005). Profiling histone modification patterns in plants using genomic tiling microarrays. Nat. Methods **2:** 213–218.

Guo, H., and Ecker, J.R. (2003). Plant responses to ethylene gas are mediated by SCF(EBF1/EBF2)-dependent proteolysis of EIN3 transcription factor. Cell **115:** 667–677.

Guo, H., and Ecker, J.R. (2004). The ethylene signaling pathway: New insights. Curr. Opin. Plant Biol. **7:** 40–49.

Guzmán, P., and Ecker, J.R. (1990). Exploiting the triple response of Arabidopsis to identify ethylene-related mutants. Plant Cell **2:** 513–523.

Hauck, P., Thilmony, R., and He, S.Y. (2003). A *Pseudomonas syringae* type III effector suppresses cell wall-based extracellular defense in susceptible Arabidopsis plants. Proc. Natl. Acad. Sci. USA **100:** 8577–8582.

He, P., Chintamanani, S., Chen, Z., Zhu, L., Kunkel, B.N., Alfano, J.R., Tang, X., and Zhou, J.M. (2004). Activation of a COI1-dependent pathway in Arabidopsis by *Pseudomonas syringae* type III effectors and coronatine. Plant J. **37:** 589–602.

Ichimura, K., Casais, C., Peck, S.C., Shinozaki, K., and Shirasu, K. (2006). MEKK1 is required for MPK4 activation and regulates tissue-specific and temperature-dependent cell death in Arabidopsis. J. Biol. Chem. **281:** 36969–36976.

Jones, J.D., and Dangl, J.L. (2006). The plant immune system. Nature **444:** 323–329.

Kosugi, S., and Ohashi, Y. (2000). Cloning and DNA-binding properties of a tobacco Ethylene-Insensitive3 (EIN3) homolog. Nucleic Acids Res. **28:** 960–967.

Li, X., Lin, H., Zhang, W., Zou, Y., Zhang, J., Tang, X., and Zhou, J.M. (2005). Flagellin induces innate immunity in nonhost interactions that is suppressed by *Pseudomonas syringae* effectors. Proc. Natl. Acad. Sci. USA **102:** 12990–12995.

Li, X., Zhang, Y., Clarke, J.D., Li, Y., and Dong, X. (1999). Identification and cloning of a negative regulator of systemic acquired resistance, SNI1, through a screen for suppressors of *npr1-1.* Cell **98:** 329–339.

Loake, G., and Grant, M. (2007). Salicylic acid in plant defence-the players and protagonists. Curr. Opin. Plant Biol. **10:** 466–472.

Lawton, K., Potter, S., Uknes, S., and Ryals, J. (1994). Acquired resistance signal transduction in *Arabidopsis* is ethylene independent. Plant Cell **6:** 581–588.

Mishina, T.E., and Zeier, J. (2007). Pathogen-associated molecular pattern recognition rather than development of tissue necrosis contributes to bacterial induction of systemic acquired resistance in Arabidopsis. Plant J. **50:** 500–513.

Mou, Z., Fan, W., and Dong, X. (2003). Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes. Cell **113:** 935–944.

Navarro, L., Zipfel, C., Rowland, O., Keller, I., Robatzek, S., Boller, T., and Jones, J.D.G. (2004). The transcriptional innate immune response to flg22. Interplay and overlap with *Avr* gene-dependent defense responses and bacterial pathogenesis. Plant Physiol. **135:** 1113–1128.

Nawrath, C., and Metraux, J.P. (1999). Salicylic acid induction-deficient mutants of *Arabidopsis* express *PR-2* and *PR-5* and accumulate high levels of camalexin after pathogen inoculation. Plant Cell **11:** 1393–1404.

Petersen, M., et al. (2000). Arabidopsis map kinase 4 negatively regulates systemic acquired resistance. Cell **103:** 1111–1120.

Potuschak, T., Lechner, E., Parmentier, Y., Yanagisawa, S., Grava, S., Koncz, C., and Genschik, P. (2003). EIN3-dependent regulation of plant ethylene hormone signaling by two Arabidopsis F box proteins: EBF1 and EBF2. Cell **115:** 679–689.

Qiu, J.L., Zhou, L., Yun, B.W., Nielsen, H.B., Fiil, B.K., Petersen, K., Mackinlay, J., Loake, G.J., Mundy, J., and Morris, P.C. (2008). Arabidopsis mitogen-activated protein kinase kinases MKK1 and MKK2 have overlapping functions in defense signaling mediated by MEKK1, MPK4, and MKS1. Plant Physiol. **148:** 212–222.

Roman, G., Lubarsky, B., Kieber, J.J., Rothenberg, M., and Ecker, J.R. (1995). Genetic analysis of ethylene signal transduction in *Arabidopsis thaliana*: Five novel mutant loci integrated into a stress response pathway. Genetics **139:** 1393–1409.

Roy, S., Garges, S., and Adhya, S. (1998). Activation and repression of transcription by differential contact: Two sides of a coin. J. Biol. Chem. **273:** 14059–14062.

Schwessinger, B., and Zipfel, C. (2008). News from the frontline: Recent insights into PAMP-triggered immunity in plants. Curr. Opin. Plant Biol. **11:** 389–395.

Shang, Y., Li, X., Cui, H., He, P., Thilmony, R., Chintamanani, S., Zwiesler-Vollick, J., Gopalan, S., Tang, X., and Zhou, J.M. (2006). RAR1, a central player in plant immunity, is targeted by *Pseudomonas syringae* effector AvrB. Proc. Natl. Acad. Sci. USA **103:** 19200–19205.

Solano, R., Stepanova, A., Chao, Q., and Ecker, J.R. (1998). Nuclear events in ethylene signaling: A transcriptional cascade mediated by ETHYLENE-INSENSITIVE3 and ETHYLENE-RESPONSE-FACTOR1. Genes Dev. **12:** 3703–3714.

Spoel, S.H., and Dong, X. (2008). Making sense of hormone crosstalk during plant immune responses. Cell Host Microbe **3:** 348–351.

Spoel, S.H., Mou, Z., Tada, Y., Spivey, N.W., Genschik, P., and Dong, X. (2009). Proteasome-mediated turnover of the transcription coactivator NPR1 plays dual roles in regulating plant immunity. Cell **137:** 860–872.

Tada, Y., Spoel, S.H., Pajerowska-Mukhtar, K., Mou, Z., Song, J., Wang, C., Zuo, J., and Dong, X. (2008). Plant immunity requires conformational charges of NPR1 via S-nitrosylation and thioredoxins. Science **321:** 952–956.

Tsuda, K., Sato, M., Glazebrook, J., Cohen, J.D., and Katagiri, F. (2008). Interplay between MAMP-triggered and SA-mediated defense responses. Plant J. **53:** 763–775.

Tusher, V.G., Tibshirani, R., and Chu, G. (2001). Significance analysis of microarrays applied to the ionizing radiation response. Proc. Natl. Acad. Sci. USA **98:** 5116–5121.

Wang, D., Amornsiripanitch, N., and Dong, X. (2006). A genomic approach to identify regulatory nodes in the transcriptional network of systemic acquired resistance in plants. PLoS Pathog. **2:** e123.

Wildermuth, M.C., Dewdney, J., Wu, G., and Ausubel, F.M. (2001). Isochorismate synthase is required to synthesize salicylic acid for plant defence. Nature **414:** 562–565.

Yamasaki, K., et al. (2005). Solution structure of the major DNA-binding domain of *Arabidopsis thaliana* Ethylene-Insensitive3-Like3. J. Mol. Biol. **348:** 253–264.

Zipfel, C., Robatzek, S., Navarro, L., Oakeley, E.J., Jones, J.D., Felix, G., and Boller, T. (2004). Bacterial disease resistance in Arabidopsis through flagellin perception. Nature **428:** 764–767.

Zuo, J., Niu, Q.W., and Chua, N.H. (2000). Technical advance: An estrogen receptor-based transactivator XVE mediates highly inducible gene expression in transgenic plants. Plant J. **24:** 265–273.

Zwiesler-Vollick, J., Plovanich-Jones, A.E., Nomura, K., Bandyopadhyay, S., Joardar, V., Kunkel, B.N., and He, S.Y. (2002). Identification of novel *hrp*-regulated genes through functional genomic analysis of the *Pseudomonas syringae* pv *tomato* DC3000 genome. Mol. Microbiol. **45:** 1207–1218.