Direct transcriptional control of the *Arabidopsis* immune receptor FLS2 by the ethylene-dependent transcription factors EIN3 and EIL1

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In plant innate immunity, the leucine-rich repeat receptor kinase FLS2 recognizes the bacterial pathogen-associated molecular pattern (PAMP) flagellin. The molecular mechanisms underlying PAMP perception are not fully understood. Here, we reveal that the gaseous phytohormone ethylene is an integral part of PAMP-triggered immunity. Plants mutated in the key ethylene-sensing protein EIN2 are impaired in all FLS2-mediated responses, correlating with reduced FLS2 transcription and protein accumulation. The EIN3 and EIN3-like transcription factors, which depend on EIN2 activity for their accumulation, directly control FLS2 expression. Our results reveal a direct role for ethylene in regulation of an innate immune receptor.

The earliest events in plant immunity concern recognition of conserved elicitors called pathogen-associated molecular patterns (PAMPs) by plasma membrane receptors. The leucine-rich repeat receptor kinase (LRR-RK) FLS2 is the receptor for the bacterial PAMP flagellin or its active epitope, represented by the peptide flg22 (1, 2). Binding of flg22 to FLS2 triggers heterodimerization with and phosphorylation of the LRR-RK BAK1 (3, 4). In addition, both FLS2 and BAK1 are able to interact with and phosphorylate the cytoplasmic kinase BIK1, which seems to act as positive regulator of the FLS2 signaling pathway (5, 6). FLS2 activation leads to activation of the MAP kinase pathways MEKK-MKK4/5-MPK3/6 and MEKK1-MKK1/2-MPK4, leading to transcription of defense-related genes through the WRKY transcription factor WRKY22/29 and WRKY25/33 (3). Recently, another pathway involving the calcium-dependent protein kinases (CDPK) 4/5/6/11 has been proposed to act in parallel to the MAPK pathways to control flg22-dependent gene expression (7). In addition, flg22 treatment triggers ion fluxes, RbohD-dependent oxidative burst, and callose deposition as well as ethylene biosynthesis (3). The development of *Arabidopsis* seedlings grown in sterile conditions is also inhibited by flg22 treatment (8). FLS2 is conserved in most plant species tested, and orthologs have been identified in *Arabidopsis*, *Nicotiana benthamiana*, tomato, and rice (1, 2). Flagellin perception is required for full immunity against bacteria, because plants deficient in FLS2 are more susceptible to adapted and nonadapted bacterial pathogens (1). Consistently, successfully pathogenic bacteria need to avoid or suppress PAMP-triggered immunity induced by flagellin (1). Interestingly, some virulence effectors from phytopathogenic bacteria do so by directly targeting FLS2 and/or BAK1 (9).

Many plant hormones, including auxin, salicylic acid (SA), jasmonates, and ethylene, impact immunity in complex and sometimes antagonistic relationships (10). Flagellin induces production of SA (11, 12) but also leads to repression of auxin perception (13). A classical response associated with PAMP treatment is an increase in the biosynthesis of the gaseous hormone ethylene (ET) (14–16). Here, we show a simple and direct link between the hormone ET and the earliest events in PAMP-triggered immunity (PTI) against pathogenic bacteria.

**Results**

To gain a better understanding of PTI signaling, we developed a forward-genetic screen to isolate *Arabidopsis* mutants impaired in the oxidative burst induced by flg22 from a collection of T-DNA mutagenized *Arabidopsis* plants (17) (Fig. S1A). Of ~35,000 *Arabidopsis* seedlings screened, 21 flagellin-insensitive (fin) mutants had reproducibly significant reduction in reactive oxygen species (ROS) intensity in response to flg22 (<60% compared with wild-type Col-0) (Fig. S1B). T-DNA–mediated mutagenesis often leads to missense mutations caused by failed T-DNA insertions (18). Indeed, sequencing of candidate genes revealed that *fin1* carries a 1-bp deletion in FLS2 (At5g46330) itself (Fig. S1 C and D), whereas *fin2* harbors a 2-bp deletion in BAK1/SEKR3 (At4g33430) (Fig. S1 E and F). The identification of FLS2 and BAK1 mutants validated our genetic screen to identify PTI components.

We focused on *fin3*, a mutant with a consistent reduction in the oxidative burst triggered by flg22 (Fig. L4 and Fig. S1B). To identify the causative mutation in *fin3*, we used a map-based cloning strategy based on reduced seedling-growth inhibition (SGI) in the presence of flg22, a phenotype also associated with the *fin3* mutation (Fig. 1 B and C). The mutation was linked to a 38.7-kb region on chromosome V that contained 12 predicted gene loci. Sequencing of candidate genes identified a 33-bp deletion in the EIN2 (At5g93280) gene, inducing an 11-amino acid deletion in its C terminus (Fig. 1D and Fig. S1G). EIN2 encodes an integral membrane protein essential for signaling in response to the gaseous plant hormone ethylene (19). Allelism tests between *fin3* and the previously characterized mutants *ein2-1* and *ein2-5* (19) confirmed that the three mutations are allelic (Table S1). Additionally, *fin3* etiolated seedlings were as impaired as *ein2-5* in the characteristic morphological triple response induced by the ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC) (19) (Fig. S2). Consistently, *ein2-1* and *ein2-5* were impaired in flg22-triggered SGI (Fig. S3). Hence, the phenotype of *fin3* is caused by a mutation in *EIN2*.

We tested if ethylene insensitivity caused by *ein2* mutations affects downstream responses triggered by flg22. In addition to the oxidative burst and SGI (Figs. 1 A–C and 2 A and D and...
Fig. S3), fin3 and ein2-5 were also impaired in callose deposition (Fig. 2B) and MAP kinase activation induced by flg22 (Fig. 2C). Notably, the number of callose deposits in ein2-5 after water treatment was higher than in water-infiltrated wild-type leaves (Fig. 2B). Because untreated ein2-5 leaves did not exhibit callose deposition (Fig. S4), this indicates that ein2-5 is more sensitive to the wounding stress caused by water infiltration. However, treatment of ein2-5 leaves with flg22 did not further increase callose deposition to levels observed in treated wild-type leaves (Fig. 2B). Notably, the resistance to the virulent bacterium **Pseudomonas syringae** pv. *tomato* DC3000 (Pto DC3000) triggered by flg22 treatment was also reduced in ein25 leaves compared with wild-type leaves (Fig. 2E), showing that the reduced flg22 responsiveness in this mutant impacts activation of innate immunity. In most assays, the effect of the ein2-5 mutation was stronger than fin3 (Fig. 2A–D), indicating that fin3 is a weaker allele than the ein2-5 null mutant. The ethylene-insensitive *etr1-1* mutant, which is impaired in ethylene perception (20), also showed reduced sensitivity to flg22 in SGI assays (Fig. S3). Thus, both ethylene perception and signaling seem to be important for responses triggered by flg22.

Our results showed that ethylene perception and signaling were required for all flg22-induced responses tested (Fig. 2A–E and Fig. S3). Because some of these responses occur within a few minutes of elicitation, we hypothesized that endogenous ethylene may control FLS2 signaling at a very early step of the pathway, possibly by controlling FLS2 itself. To test this hypothesis, we measured the steady-state levels of FLS2 protein in immmunoblots. Strikingly, FLS2 levels were dramatically reduced in ein2-5 and ein2-1 (Fig. 3A and Fig. S5A). The reduction of FLS2 protein levels correlated with a similar reduction of steady-state FLS2 transcript accumulation in ein2-5 and ein2-1 (Fig. 3B and Fig. S5B). A comparable reduction in FLS2 transcript and protein accumulation was also observed in *etr1-1* (Fig. 3A and B). Consistent with our previous results (Fig. 2A–D) and the reduced FLS2 expression (Fig. 3A and B), the transcript levels of FLS2 and SIRK/FRK1 after flg22 treatment were also reduced in ein2-5 (Fig. S6A and B). Thus, the impaired flg22 sensitivity of ein2-5 and *etr1-1* is likely caused by reduced amount of FLS2 proteins. Our results show that both ethylene perception and signaling are crucial for FLS2 expression.

We investigated how ethylene could influence FLS2 expression. EIN2 is the only gene whose recessive loss-of-function mutation leads to complete ethylene insensitivity in the triple-response assay (19). EIN2 controls the accumulation and subsequent activity of the functionally redundant EIN3 and EIN5-like (EIL) transcription factors in response to ethylene (21, 22). We, therefore, tested if EIN3 could regulate FLS2 transcription directly. In silico motif analysis of the FLS2 promoter revealed the presence of nine potential EIN3/EILs binding sites (23, 24) (Fig. S7), suggesting that EIN3 may bind to the promoter of the FLS2 gene to influence its transcription. Regions of the *Arabidopsis* genome associated with EIN3 were identified by ChiP using a purified EIN3 antibody (21) and followed by Illumina sequencing (ChiP-Seq). Notably, EIN3 binds to two positions in the FLS2 promoter in wild-type etiolated seedlings treated with ethylene (Fig. 4A). EIN3 binding to these regions was absent in chromatin purified from the loss-of-function allele ein3-1, proving the specificity of the enrichment (Fig. 4A). Relative enrichments of EIN3 binding sites in the FLS2 promoter after immunoprecipitation with the EIN3 antibody were further confirmed by quantitative PCR using chromatin from light-grown seedlings (Fig. 4B). Comparison of the EIN3 binding sites experimentally determined for the *ERF1* (23) and *EBF2* (25) promoters reveals a close homology with the two potential sites identified in the FLS2 promoter (Fig. 4C). Whereas EIN3 is able to bind to the FLS2 promoter, FLS2 transcript levels were not significantly reduced in ein3-1 (Fig. 4D). The effect of single mutations in EIN3 and EIL transcription factors is often hindered by their homology and consequent functional redundancy (17, 26, 27). Indeed, simultaneous mutations in EIN3 and EIL1 accentuated the effect on FLS2 expression, with the double mutant *ein3-1 eil1-1* accumulating as little FLS2 transcript as the *ein2-5* mutant (Fig. 4D). Consequently, *ein3-1 eil1-1* plants were impaired in flg22-triggered oxidative bursts to a level comparable with ein2-5 plants (Fig. 4E).

To test if endogenous ethylene could control early responses independently of FLS2 transcription, we measured the oxidative burst induced by the general phosphatase inhibitor calycin A
that stimulates early PAMP responses downstream and independently of pattern-recognition receptors (PRRs) (4, 28). In contrast to the flg22-induced oxidative burst, the burst induced by calyculin A was not inhibited in fls2 or ein2-5 seedlings (Fig. S8). The oxidative burst induced by both elicitors was, however, completely abolished in rbohD seedlings lacking the NADPH oxidase required for PAMP-induced oxidative burst in Arabidopsis. Thus, these experiments suggest that the reduced ROS burst triggered by flg22 in ethylene perception and signaling mutants is caused by reduced FLS2 expression and is not an indirect effect on downstream responses.

Together, these results reveal that endogenous ethylene controls FLS2 expression transcriptionally through direct binding of the transcription factor EIN3 and potentially, EIL1 to the FLS2 promoter.

Discussion

An inherent difficulty in interpreting the specific contributions of ethylene to plant immunity is its role in multiple different physiological processes, often through cross-talk with other hormones. For example, ethylene controls senescence, symptom development, and abiotic resistance together with abscisic acid and gibberellic acid and resistance to insects and necrotrophic pathogens in association with jasmonic acid (29, 30). Previous reports show the role of ethylene in disease symptom development and susceptibility to bacteria conflict (29, 30). Recently, EIN3 and EIL1 were shown to repress biosynthesis of the immune hormone salicylic acid (SA) (31). Consequently, ein2-1 and ein3-1 eil1-1 plants exhibited enhanced resistance to the pathogenic bacterium P. syringae pv. tomato (Pto) DC3000, suggesting that they over-accumulate (SA) (31). However, a previous study showed that ethylene-insensitive mutant plants were more tolerant to bacteria (reduced symptoms), but no decrease in bacterial numbers could be observed (32, 33); we observed that Pto DC3000 infiltrated at low dose (105 cfu/mL) multiplied to lower levels in ein2-5 and ein2-1 leaves than in wild-type leaves (Fig. 2E) (34), suggesting that EIN2 may indeed play an additional role as a negative regulator of SA-mediated biosynthesis or signaling. In addition, ein3-1 eil1-1 plants exhibited deregulated callose deposition and defense gene expression in response to flg22 (31). In our conditions, we could confirm overaccumulation of SA (Fig. S9A) and increased expression of the SA-induced PRI gene in ein3-1 eil1-1 soil-grown plants (Fig. S9B). However, we did not detect changes in SA levels in the single mutants ein3-1 or eil1-1 (Fig. S9A). Furthermore, the triple mutant ein3-1 eil1-1 sid2-2 that exhibits wild-type SA levels (31) accumulated as little FLS2 mRNA as the double mutant ein3-1 eil1-1 (Fig. S9C). Thus, the effect of the ein3-1 eil1-1 mutation on FLS2 gene is not related to alterations in SA levels. In agreement with our findings that ethylene controls FLS2 expression and thus, flg22

Fig. 2. Ethylene signaling is required for flg22-induced responses. (A) Total ROS production triggered by 100 nM flg22 in Col-0, fin3, and ein2-5 mutants. Values are expressed as percentage of ROS obtained with Col-0 for 40 min and are mean ± SE (n = 20). (B) Callose deposition triggered in leaves 24 h after infiltration with water or 1 μM flg22 in Col-0, fin3, and ein2-5 plants. Results showing quantification of callose deposits (foci/mm2) are mean ± SE (n = 4). For ein2-5, the same letter indicates statistically nonsignificant (one-way ANOVA; P > 0.05) differences between samples. (C) Activation profile of the MAP kinases MPK3 and MPK6 in response to treatment with water or 100 nM flg22 in Col-0, fin3, and ein2-5 mutants. Arrowheads indicate phosphorylated MPK6 and MPK3. Blots stained with colloidal Coomassie Blue (CCB) are presented to show equal loading. (D) Growth inhibition of Col-0, fin3, and ein2-5 seedlings treated with different concentrations of flg22 for 12 d. Values are mean ± SE (n = 12). (E) Growth of Pto DC3000 on Col-0, fls2, and ein2-5 plants pretreated with water or 1 μM flg22 for 24 h and then syringe-infiltrated with 105 cfu/mL of bacteria. Bacterial growth was determined 2 d postinoculation. Values are mean ± SE (n = 8). Statistical significance by comparison with Col-0 was assessed using one-way ANOVA followed by Dunnett’s test. *P < 0.05; **P < 0.01; ***P < 0.001. Similar results were observed in at least three independent experiments.

Fig. 3. Endogenous ethylene signaling regulates FLS2 expression. (A) Western blot showing accumulation of FLS2 proteins in Col-0, ein2-5, and etr1-1 13-d-old seedlings. The fls2 mutant was used as a negative control for FLS2 detection. Thirty micrograms of total protein were loaded in each lane. Blots stained with CCB are presented to show equal loading. Similar results were observed in at least three independent experiments. (B) Quantitative RT-PCR analysis of FLS2 expression in Col-0, etr1-1, and ein2-5 untreated 13-d-old seedlings. Transcript levels are normalized to the U-box housekeeping gene At5g15400 and are presented as relative to untreated Col-0. Similar results were observed in at least three independent experiments. Statistical significance by comparison with untreated Col-0 was assessed using one-way ANOVA followed by Dunnett’s test. ***P < 0.001.
induced by pre-
Flg22 peptide was purchased from Peptron and solubilized in

Flg22 response. In etiolated seedlings, ethylene production and signaling are all impaired in flg22-induced responses and that this always correlates with a corresponding reduction in FLS2 transcript levels. In addition, we revealed that the key transcription factor EIN3, which depends on ethylene perception and signaling for its accumulation and activity, binds directly to the FLS2 promoter. These results showed that endogenous ethylene controls the expression of the PRR FLS2 at the transcriptional level.

Our findings that the oxidative burst triggered by the elicitor-mimic calyculin A is not reduced in ein2-5 mutants indicate that the reduced flg22 responses in ethylene-insensitive mutants is caused by reduced FLS2 expression and not an indirect effect on downstream responses. However, future experiments, in which FLS2 gene expression is constitutively in ethylene-insensitive mutants, are required to ascertain the causal relationship between decreased FLS2 expression and impaired FLS2-mediated responses.

Flg22 induces the MAP kinases MPK3 and MPK6 (39, 40) as well as ethylene production (16). These same MAP kinases phosphorylate the ethylene biosynthetic enzymes ACC synthases (ACS) 2 and 6 as well as EIN3, leading to its stabilization (41, 42). We, therefore, hypothesize a simple feedback model in which ethylene produced in response to flg22 treatment could maintain FLS2 levels in a positive feedback loop. This mechanism would be especially effective to maintain the plasma membrane FLS2 pool after internalization after flagellin binding and receptor activation (43). In the absence of flagellin, endogenous ethylene ensures a constitutive level of FLS2 expression. On flg22 binding, FLS2 activates MPK6 that, in turn, phosphorylates ACS2/6 and further leads to EIN3 stabilization, resulting in increased ethylene production and signaling. Congruently, flg22 perception leads to EIN3 accumulation (31). This model may explain the long-established observation that PAMP perception triggers increased ethylene biosynthesis (14–16). Our results show an unexpected layer of regulation by ethylene to maintain optimal levels of innate immune receptors.

Materials and Methods
Plant Material and Growth Conditions. Arabidopsis thaliana ecotype Columbia (Col-0) was used as the wild-type control. Arabidopsis plants used in this study were grown as one plant per pot at 20–21 °C with a 10-h photoperiod in environmentally controlled chambers or on plates containing Murashige and Skoog (MS) medium (including vitamins) (Duchefa) and 1% sucrose supplemented with 8% agar for the first 5 d at 22 °C and with a 16-h photoperiod. The first set of 10,000 T-DNA SALK lines (17) was obtained from the Nottingham Arabidopsis Stock Center. The mutant lines used in this study are: fls2 (SALK_093905), cerk1-2 (GK-096F09), rbohD, ett1-1, ein2-1 ein2-5, ein3-1, ett1-1, sid2-2, ein3-1 ett1-1, and ein3-1 ett1-1 sid2-2.

Elicitor. Flg22 peptide was purchased from Peptron and solubilized in sterile water.

Identification of fn Mutants. To identify flg22 insensitive (fn) mutants, we screened ~35,000 Arabidopsis seedlings from the first set of 10,000 T-DNA SALK lines produced in the Col-0 background (17). Surface-sterilized seeds

**Responsiveness**, the resistance to Pto DC3000 induced by pretreatment with flg22 was reduced in ein2-5 and ein2-1 leaves (Fig. 2E) (34, 35).

Ethylene perception and signaling were recently proposed to be required for callose deposition in response to flg22 (36). However, the callose deposition observed in ein2-5 after water infiltration (Fig. 2B) excludes a requirement for EIN2 in callose biosynthesis. Furthermore, the reduced FLS2 levels in ein2-5 (Fig. 3A) explain the decreased callose deposition observed in this mutant after flg22 treatment (Fig. 2B). Our findings also explain the requirement for ethylene signaling in disruption of the association between MPK6 and the transcription factor ERF104 in response to flg22 and definitively place endogenous ethylene signaling upstream of MAP kinase activation by PAMPs (37, 38).

The data presented here reveal a surprisingly simple and direct mechanism involving ethylene that ensures optimal levels of an immune receptor. Previous reports have highlighted the complexity of ethylene signaling and its roles in development of disease symptoms, induction of antimicrobial genes as a secondary response, and cross-talk with other hormones (29). Here, we showed that mutants impaired in ethylene perception and signaling are all impaired in flg22-induced responses and that this always correlates with a corresponding reduction in FLS2 transcript levels. In addition, we revealed that the key transcription factor EIN3, which depends on ethylene perception and signaling for its accumulation and activity, binds directly to the FLS2 promoter. These results showed that endogenous ethylene controls the expression of the PRR FLS2 at the transcriptional level.

Fig. 4. EIN3/EIL1 directly control FLS2 expression at the transcriptional level. (A) Alignment of ChIP-Seq reads across the FLS2 (At5g46330) gene and promoter region as shown with Anno-1 viewer. Col-0 or ein3-1 etiolated seedlings were grown in hydrocarbon-free air for 3 d, treated with 10 ppm ethylene gas for 4 h, and then used in ChIP-Seq analysis using an anti-EIN3 polyclonal antibody. The proximal and distal regions with significant enrichment are highlighted by red brackets and dashed lines. Results from two independent experiments are presented. Reads are normalized for each sample. Gene annotation is depicted at the top; filled boxes and open boxes represent the 5′- and 3′-untranslated regions and exons of the gene, respectively. (B) Enrichment of the indicated EIN3-associated DNA fragments after ChIP-PCR. Chromatin from wild-type Col-0 and ein2-1 light-grown seedlings was immunoprecipitated with an anti-EIN3 polyclonal antibody. Enrichment of associated DNA fragments was verified by qPCR using specific primers and is presented as relative to Col-0. (C) Sequence alignment of EIN3 binding sites in ERF1, EBF2, and FLS2 promoters. EIN3 binding sites in the promoters of ERF1 (23) and EBF2 (25) are presented. Indicated by ChIP-Seq, the two potential binding sites of EIN3 to the FLS2 promoter are also shown. (D) Quantitative RT-PCR analysis of FLS2 expression in Col-0, ein2-5, ein3-1, eil1-1, and ein3-1 eil1-1 seedlings. Transcript levels are normalized to the U-box housekeeping gene and are presented as relative to Col-0. Data are representative of one of two experiments. (E) Total ROS production triggered by 100 nM flg22 in Col-0, ein2-5, ein3-1, eil1-1, and ein3-1 eil1-1 mutants. Values are expressed as percentage of ROS obtained with Col-0 for 40 min and are mean ± SE (n = 20). Statistical significance by comparison with (B) ein3-1 and (C) Col-0 was assessed using one-way ANOVA followed by Dunnett’s test. *p < 0.05; **p < 0.01; ***p < 0.001. Similar results were observed in at least three independent experiments.
were individually dispensed in 96-well plates containing 1× Gamborg B5 medium (including vitamins) (Duchefa) supplemented with 1% sucrose, pH 5.7. After 2 d of stratification at 4 °C, plates were transferred to light for 9 d. Liquid media were then removed and replaced by assay solution containing 10 μg/mL peroxidase from horseradish Type VI-A (Sigma), 100 μM luminol (Sigma), and 100 mM fig22. ROS production was measured immediately and during the 15-min period using a Variskan microplate luminometer (Thermo Scientific). Seedlings with a ROS production comparable with fig22- insensitive fls2 mutant were rescued by rinsing in water and transferred to the greenhouse. Three and four weeks later, production of oxidative burst was tested again with two leaf discs and 100 nM fig22 as described in ref. 44. SALK lines were considered as fln candidates when they produce repeatedly, in the leaf disk assay, less than 60% of the ROS production obtained with wild-type Col-0.

**Map-Based Cloning.** The fln3 mutation (in Col-0 background) was identified by map-based cloning as described in SI Materials and Methods.

**Measurement of Reactive Oxygen-Species Generation.** Oxidative-burst measurement was performed as previously described (44). ROS was elicited with 100 nM fig22, and elicitation in the absence of any PAMP (water treatment) was included in all experiments as negative control. Twenty leaf discs from 10 5-wk-old plants were used for each condition. ROS was also triggered by 5 mM MES, pH 6.5, containing whole 7- to 8-d-old seedlings grown in 1× Gamborg B5 liquid medium (including vitamins) supplemented with 1% sucrose (pH 5.7). Luminescence was measured over time using an ICCD photon-counting camera (Photek).

**Seedling-Growth Inhibition and Callose Assays.** Quantitative seedling-growth inhibition was assessed using 12 seedlings per condition as described in ref. 45. Callose deposition analysis was performed as described in ref. 46.

**MAP Kinase Activation.** MAPK assays were performed on six 2-wk-old seedlings grown in liquid medium. Seedlings were then elicited with 100 nM fig22 or water for 5, 10, or 30 min and frozen in liquid nitrogen. MAPK activation was monitored by Western blot with antibodies that recognize the dual phosphorylation of the activation loop of MAPK (pTepY). Phospho-p44/42 MAP kinase (p44/42) rabbit monoclonal antibodies from Cell Signaling were used according to the manufacturer’s protocol. Blots were stained with colloidal Coomassie blue (CCB) to verify equal loading.

**Induced Resistance to Bacteria.** Induced resistance assays were realized as described in ref. 34. Briefly, water or 1 μM fig22 was infiltrated with a needleless syringe into leaves of 5-wk-old Arabidopsis plants. After 24 h, the same leaves were syringe-infiltrated with 10−4 M Cs of P. syringae pv. tomato DC3000. Bacterial growth was determined 2 d postinoculation.

**RNA Isolation and Quantitative RT-PCR.** Total RNA was extracted from six 2-wk-old seedlings grown in liquid medium using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. RNA samples were treated with Turbo DNA-free DNase (Ambion) and quantified with a Nanodrop spectrophotometer (Thermo Scientific). cDNA synthesis and qRT-PCR were realized as described in SI Materials and Methods.

**Protein Extraction and Immunoblot Assays.** To prepare samples for examining the accumulation of PRR proteins, five leaf discs (0.38 cm² each) from soil-grown plants or six 13-d-old seedlings grown in sterile conditions were harvested and ground in liquid nitrogen. Total protein crude extracts were prepared with 100 μL of extraction buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 2 mM DTT, 0.5 mM PMSE, 0.1% glycerol, 1% v/v vol protease inhibitor mixture for plant cell [Sigma]). The lysates were cleared by centrifugation at 16,000 × g for 5 min. The supernatants were collected, and protein concentrations were determined by Bradford quantification (Bio-Rad) according to the manufacturer’s indications; 30 μg of total proteins were separated on SDS/PAGE gels. FLS2 was detected with 1:1,000 rabbit anti-FLS2 polyclonal antibodies (44). A peroxidase-conjugated goat anti-rabbit IgG (Sigma) was used as secondary antibody.

**ChIP-Seq and ChIP-PCR.** ChIP was performed as described in ref. 47 with modifications using Col-0 or ein3-1 etiolated seedlings grown for 3 d in hydrocarbon-free air-treated for 4 h with 10 ppm ethylene gas followed by sequencing as per illumina specifications. Sequencing data were visualized using the Anno-J viewer (http://www.anno.org). Enrichment values have been determined using PeakSeq (48). ChIP was performed again using 13-d-old leaves from Col-0, ein3-1, and ein3-1 seedlings followed by quantitative PCR. Actin was used as internal control for normalization. Primers used for quantitative PCR are as follows: FL52 (At5g46320:1251 binding site, forward 5′-CAAGCTTCAAGAAAATACATGTAACG-3′ and reverse 5′-GATTTGGAACATCCTATCCACC-3′); FL52 (At5g46320:267 binding site, forward 5′-CAAGCTCTAATACAAATGTCG-3′ and reverse 5′-GGTGTTGAAGGGCAAAGAGCC-3′); ACT5 (At5g09810) forward 5′-CGTTGCTTCTTCTTCTGTGAT-3′ and reverse 5′-AGGACACCGATCTAGAGCTACCGT-3′ (49). In silico motif analysis of the FL52 promoter for the presence of primary ethylene response elements (AYGWAYCT) was realized using the fuzznuc software (http://embossgui.sourceforge.net/netdemo/fuzznuc.html).

**SA Measurement.** SA extraction followed by liquid chromatography–MS quantification was realized using 5-wk-old plants as described in ref. 50.

**Statistical Analysis.** Statistical significances based on t test and one-way ANOVA analyses were performed with Prism 5.01 software (GraphPad Software).

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Science
Nicotiana benthamiana
Xanthomonas
Pseudomonas
innate immune response.
Arabidopsis thaliana
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Plant Cell
pathogens.
Mol Plant Microbe Interact
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Nature
415:977
innate immunity.
| Mol Cell
infected with virulent and avirulent
428:764
Proc Natl Acad
F box proteins: EBF1 and EBF2.
EIN3 binding F-Box proteins EBF1 and EBF2
expression to negatively regulate
Genes Dev
115:667
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SALICYLIC ACID INDUCTION DEFICIENT2
241:1086
Plant J
451:789
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