Flagellin induces innate immunity in nonhost interactions that is suppressed by *Pseudomonas syringae* effectors

Xinyan Li*†, Huiqiong Lin‡, Weiguo Zhang‡, Yan Zou‡, Jie Zhang‡, Xiaoyan Tang†, and Jian-Min Zhou*‡§

‡National Institute of Biological Sciences, Beijing 102206, China; *Department of Plant Pathology, Kansas State University, Manhattan, KS 66502; and †Cytogenetics Institute, Nanjing Agricultural University, Nanjing 210095, China

Edited by Frederick M. Ausubel, Massachusetts General Hospital, Boston, MA, and approved June 30, 2005 (received for review March 24, 2005)

***Arabidopsis NONHOST1* (*NHO1*) is required for limiting the *in planta* growth of nonhost *Pseudomonas* bacteria but completely ineffective against the virulent bacterium *Pseudomonas syringae* pv. *tomato* DC3000. However, the molecular basis underlying this observation remains unknown. Here we show that *NHO1* is transcriptionally activated by flagellin. The nonhost bacterium *P. syringae* pv. *tabaci* lacking flagellin is unable to induce *NHO1*, multiplies much better than does the wild-type bacterium, and causes disease symptoms on *Arabidopsis*. DC3000 also possesses flagellin that is potent in *NHO1* induction, but this induction is rapidly suppressed by DC3000 in a type III secretion system-dependent manner. Direct expression of DC3000 effectors in protoplasts indicated that at least nine effectors, HopS1, HopAI1, HopAF1, HopT1-1, HopT1-2, HopAA1-1, HopF2, HopC1, and AvrPto, are capable of suppressing the flagellin-induced *NHO1* expression. One of the effectors, HopAI1, is conserved in both animal and plant bacteria. When expressed in transgenic *Arabidopsis* plants, HopAI1 promotes growth of the nonpathogenic *hrpL*$^-$ mutant bacteria. In addition, the purified phytotoxin coronatine, a known virulence factor of *P. syringae*, suppresses the flagellin-induced *NHO1* transcription. These results demonstrate that flagellin-induced defenses play an important role in nonhost resistance. A remarkable number of DC3000 virulence factors act in the plant cell by suppressing the species level defenses, and that contributes to the specialization of DC3000 on *Arabidopsis*.**

type III secretion system | virulence | pathogen-associated molecular patterns | basal defense | coronatine

Nonhost resistance refers to resistance shown by an entire plant species to a specific parasite (1). This resistance is expressed by every plant toward the majority of potential phytopathogens and differs from the cultivar-level resistance conditioned by gene-for-gene interactions (2, 3). Plant defenses can be induced by "general elicitors" of pathogen or plant origin, including oligosaccharides, lipids, polypeptides, and glycoproteins (4). However, a role of these elicitors in plant disease resistance in a natural setting is often difficult to establish, because plants' responses to elicitors do not differentiate resistant and susceptible plants. Many of the elicitors are now known as pathogen-associated molecular patterns (PAMPs). The best-characterized PAMP known to activate innate immunity in plants is flagellin from *Pseudomonas* bacteria (5). A conserved N-terminal peptide of flagellin, flg22, is a potent elicitor of defense responses in tomato and *Arabidopsis* (5, 6). In *Arabidopsis*, flg22 is perceived by FLS2, a receptor-like kinase that activates downstream events through a MAP kinase cascade (7, 8). Pretreatment of *Arabidopsis* with flg22 peptide not only globally induces defense gene expression, but also protects plants from subsequent infection of the virulent DC3000 (9). *Arabidopsis* plants lacking *FLS2* exhibit enhanced disease susceptibility to DC3000 under certain circumstances (9). Although these studies elegantly demonstrated the functional significance of flagellin-sensing in plant defense, whether flagellin-signaling plays a role in the species level resistance remains unknown.

The bacteria enter plants through natural openings such as stomata or wounds and proliferate in the intercellular spaces. A major bacterial pathogenesis mechanism is mediated by the so-called type-III secretion system (TTSS), through which Gram-negative bacteria inject a repertoire of effectors into host cells (10). Type III effectors play an important role in bacterial pathogenesis. In *Pseudomonas syringae*, a growing number of effector genes, such as *avrRpt2*, *avrRpm1*, *virPphA(hopAB1)*, *avrPto*, and *hopAB2(avrPtoB)*, are known to contribute to virulence (11–16). *avrRpt2*, for example, suppresses plant PR gene expression and interferes with the RPM1-specified resistance (11). *avrPtoB*, *hopX1(avrPphE$_{pto}$)*, *hopAM1(avrPpiB$_{pto}$)*, *hopAO1(hopPtoD2)*, *hopE1(hopPtoE)*, *hopF2(hopPtoF)*, *hopF1(avrPphF)*, and *hopN1*(*hopPtoN*) all appear to suppress cell death in plants (17, 10). In addition to type III effectors, certain *P. syringae* strains, including DC3000, produce the phytotoxin coronatine, which also plays a role in bacterial virulence (18). A role of TTSS or coronatine in overcoming nonhost resistance has not been examined closely.

In previous studies, we showed that the *Arabidopsis NHO1* gene is required for resistance to multiple strains of nonhost *P. syringae*, but completely ineffective against DC3000 (19). Interestingly, *NHO1* transcripts are induced by the nonhost strains, but suppressed by DC3000 (20). This suppression is apparently of functional significance, because plants overexpressing *NHO1* exhibit enhanced resistance to DC3000 (20).

Here, we show that the flg22 peptide strongly induces the transcription of *NHO1*. A *P. syringae* pv. *tabaci* (*Ptab*) strain, to which *Arabidopsis* is a nonhost plant, induces *NHO1* in a flagellin-dependent manner. A *Ptab* strain lacking the flagellin gene *fliC* elicits disease symptoms and multiplies in *Arabidopsis* plants, demonstrating that flagellin signaling contributes to nonhost resistance. In contrast to nonhost bacteria that give a prolonged induction of *NHO1*, DC3000 only transiently induces *NHO1* transcription, also in a flagellin-dependent manner. Although the wild-type DC3000 rapidly suppresses the *NHO1* induction, DC3000 mutant strains defective in TTSS are diminished in their ability to suppress *NHO1*. Expression of the DC3000 effectors HopS1, HopAI1, HopAF1, HopT1-1, HopT1-2, HopAA1-1, HopF2, HopC, and AvrPto in the plant cell blocks the *NHO1* induction by flg22. In addition, purified coronatine suppresses the flg22- and *P. syringae* pv. *phaseolicola* (*Pph*)-induced *NHO1* expression. Furthermore, expression of HopAI1 in transgenic plants promotes nonpathogenic bacterial growth. Together, these results demonstrate the importance of flagellin-induced innate immunity mechanism in nonhost resis-

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: PAMP, pathogen-associated molecular pattern; TTSS, type-III secretion system; ddH_2O, double distilled H_2O; *Ptab, Pseudomonas syringae* pv. *tabaci*; *Pph*, *P. syringae* pv. *phaseolicola*.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. DQ077692).

§To whom correspondence should be addressed. E-mail: zhoujianmin@nibs.ac.cn.

tance and a role of DC3000 virulence factors in suppressing the flagellin-induced innate immunity.

Materials and Methods

Construction of *NHO1–LUC* Reporter Line. A 1.8-kb *NHO1* promoter sequence was PCR-amplified from Col-0 genomic DNA with the following primers: 5′-CAGTCGACTTCCTTACAGTCCAGACATG-3′ and 5′-TTCCCGGGGGTAAAGGTGAAGAACGATGCT-3′. The PCR product was digested with SalI and SmaI and cloned into a modified pBI121 vector with the *LUC* reporter gene (21). The *NHO1–LUC* construct was introduced into *Agrobacterium tumefaciens* strain GV3101 and transformed into Col-0 plants by floral-dipping (22). The T4 progeny of a selected homozygous transgenic line with a single insertion was used for all experiments.

Bacterial Strains and Bacterial Growth Assay. Bacterial strains used in this study include DC3000, *Pph* NPS3121 (19), *Ptab* 6505 wild-type and 6505 *fliC*$^-$ mutant (23), DC3000 *hrpL*$^-$ mutant (24), DC3000 *hrpA*$^-$ and *hrcC*$^-$ mutants (25, 26), and DC3000 *fliC*$^-$ mutant (previously referred to as *flaA*; ref. 27). Bacteria were grown overnight at room temperature in King's medium B with appropriate antibiotics, precipitated, washed twice with double distilled H_2O (ddH_2O) and diluted to the desired concentration with ddH_2O for plant inoculation. Bacteria used for growth assay was diluted to 10^5 colony-forming units (cfu)/ml and syringe-infiltrated into young and fully expanded *Arabidopsis* leaves. All experiments were repeated at least twice with similar results.

Luciferase Activity Assay. Bacterial cultures used for luciferase activity assay were diluted with 0.2 mM luciferin to 10^8 cfu/ml and syringe-infiltrated into *Arabidopsis* leaves. The leaves were removed from plants at the indicated time points and sprayed with 1 mM luciferin containing 0.01% Triton X-100. Luminescence images were captured by using a low-light imaging system, and relative luciferase activity was calculated with WINVIEW software (Roper Scientific, Trenton, NJ) (21).

Flagellin and Coronatine Treatment. Polypeptides containing 22 conserved N-terminal residues of flagellin from *P. aeruginosa*, *Ptab*, and *Agrobacterium tumefaciens* were synthesized by Bio-Synthesis (Lewisville, TX) as the following: flg22$^{P.aeruginosa}$, QRLSTGSRINSAKDDAAGLQIA; flg22$^{A.tumefaciens}$, ARVSSGLRVGDASDNAAYWSIA; and flg22$^{P.s.tabaci}$, TRLSSGLKINSAKDDAAGLQIA. Flg22 peptides were dissolved in ddH_2O and diluted to 1 μM with 0.2 mM luciferin before inoculation. Coronatine (kindly provided by Carol Bender, Oklahoma State University, Stillwater) was dissolved in ddH_2O and diluted to 100 ng/ml with 0.2 mM luciferin before inoculation.

Construction of Effector Gene Expression Plasmids. A transient expression vector pUC19-35S-FLAG-RBS containing the cauliflower mosaic virsu 35S promoter, 3× FLAG, and a *Rubisco Small Subunit* terminator (Y. Zou and J.M.Z. unpublished results; GenBank accession no. DQ077692) was used for transient expression of effector genes in protoplasts. The effector genes were PCR-amplified with primers listed in Table 1, which is published as supporting information on the PNAS web site. After restriction digestion, the *avrPto* PCR product was inserted between the XhoI and SpeI sites of pUC19-35S-3×FLAG-RBS, resulting in the 35S-AvrPto construct. Other effector genes were inserted between XhoI and *Csp45*I of pUC19-35S-FLAG-RBS, resulting in 35S-Effector-FLAG constructs.

Protoplast Transfection Assay. Protoplasts were isolated from 6-week-old *NHO1–LUC* plants according to Sheen (http://genetics.mgh.harvard.edu/sheenweb). Protoplasts were transfected with either an effector construct or the empty vector, and incubated in 0.4 M mannitol and 1 μM flg22$^{P.s.tabaci}$ for 12 h. LUC activity was measured after adding 50 μM luciferin to the transfected protoplasts.

Construction of Estradiol-Inducible *hopAI1* Expression Plants. The HopAI1-FLAG fragment was excised from the 35S-HopAI1-FLAG plasmid with XhoI and SpeI and inserted into pER8 (28). The construct was transformed into *Arabidopsis* plants (Col-0) by *Agrobacterium*-mediated transformation. Transgenic plants were selected on Murashige and Skoog plates containing hygromycin. For *hopAI1* induction, plants were sprayed with 25 μM estradiol containing 0.02% silwet L-77.

Supporting Information. For further details, see *Supporting Text*, Table 1, and Figs. 7–9, which are published as supporting information on the PNAS web site.

Results

Flagellin Induces *NHO1* Transcription. To further investigate the regulation of *NHO1* expression in response to *Pseudomonas* bacteria, an *NHO1–LUC* reporter line was constructed. Consistent with the expression of endogenous *NHO1* mRNA (20), the *NHO1–LUC* expression in a homozygous reporter line was strongly induced by *Pph*, but not DC3000 (Fig. 1*a*). Detailed analysis revealed a transient *NHO1–LUC* induction 3 h after DC3000-inoculation, but the *NHO1–LUC* expression returned to the baseline by 12 h (Fig. 1*b*). In contrast, *Pph* induced a strong and sustained expression of *NHO1–LUC*. The strong induction by nonhost bacteria is not strain-specific, because another nonhost strain *Ptab* also induced *NHO1–LUC* to a high level (Fig. 2*a*).

We previously hypothesized that a PAMP derived from the nonhost *Pseudomonas* bacteria induces the expression of *NHO1* (20). Flagellin is a well known PAMP that induces innate immune responses in plants and animals. Therefore, we tested whether flagellin induces the *NHO1–LUC* reporter gene. flg22 peptides corresponding to *P. aeruginosa*, *A. tumefaciens*, and *P. syringae* pv. *tabaci* were tested for their ability to induce *NHO1–LUC.* Fig. 2 shows that the active peptide flg22$^{P.aeruginosa}$ was fully capable of inducing *NHO1–LUC.* Flg22$^{P.s.tabaci}$ was similarly active in *NHO1–LUC* induction (see Fig. 6*a*). In contrast, flg22$^{A.\ tumefaciens}$, which is inactive in plant immune response induction (5), was unable to induce *NHO1–LUC* (Fig. 1*c*).

Flagellin Is Required for *NHO1* Induction and Resistance in Nonhost Interaction. If flagellin is required for the *NHO1* induction by a nonhost bacterium, then bacteria lacking flagellin should be defective in *NHO1* induction. A *Ptab* mutant strain lacking the flagellin gene *fliC*$^-$ (23) poorly induced the *NHO1–LUC* expression (Fig. 2*a*), indicating that flagellin is largely responsible for the observed induction of *NHO1* by this bacterium. To test whether flagellin contributes to nonhost resistance in *Arabidopsis* plants, the *fliC*$^-$ mutant was compared with the wild-type *Ptab* for disease symptoms and bacterial growth *in planta.* Fig. 2*b* shows that *fliC*$^-$ caused visible disease symptoms on *Arabidopsis*. In contrast, the wild-type bacterium caused no visible symptoms. The mutant bacteria multiplied at least 10-fold 4 days after inoculation, whereas the wild-type *Ptab* failed to multiply in the 4-day period (Fig. 2*c*). Together, these data demonstrate that flagellin is a major PAMP responsible for the induction of *NHO1* and resistance to this nonhost *P. syringae* bacterium.

Transient Induction of *NHO1* by DC3000 Requires Flagellin. The possibility that transient *NHO1–LUC* induction by the wild-type DC3000 depends on flagellin was also tested. The *fliC*$^-$ mutant of DC3000 failed to induce *NHO1–LUC* at any tested time point after inoculation (Fig. 3*a*). In addition, wild-type DC3000 bacteria killed by exposure to kanamycin before inoculation also induced a strong and sustained *NHO1–LUC* expression, whereas the *fliC*$^-$ bacteria

PLANT BIOLOGY

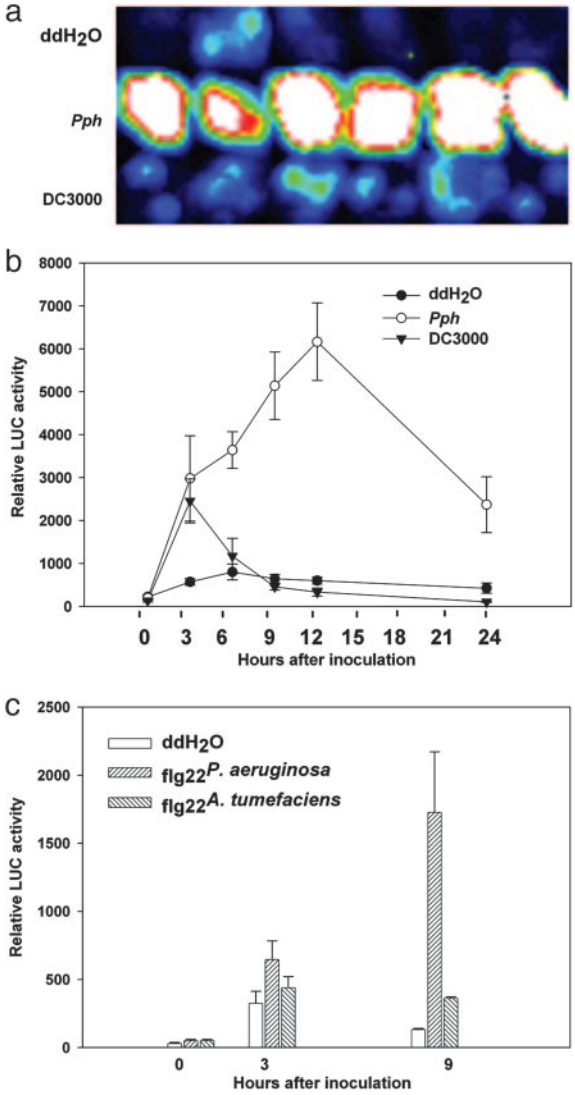

Fig. 1. *NHO1* is transcriptionally induced by nonhost bacteria and flagellin. (*a*) A luciferase image of *NHO1–LUC* transgenic leaves inoculated with water, nonhost strain *Pph*, or virulent strain DC3000 for 24 h. (*b*) Time course of *NHO1–LUC* expression in plants inoculated with water, *Pph*, or DC3000 bacteria. (*c*) *NHO1–LUC* activity of plants inoculated with 1 μM flg22$^{P.aeruginosa}$ or flg22$^{A.tumefaciens}$ at the indicated hours. The experiments were repeated numerous times with similar results.

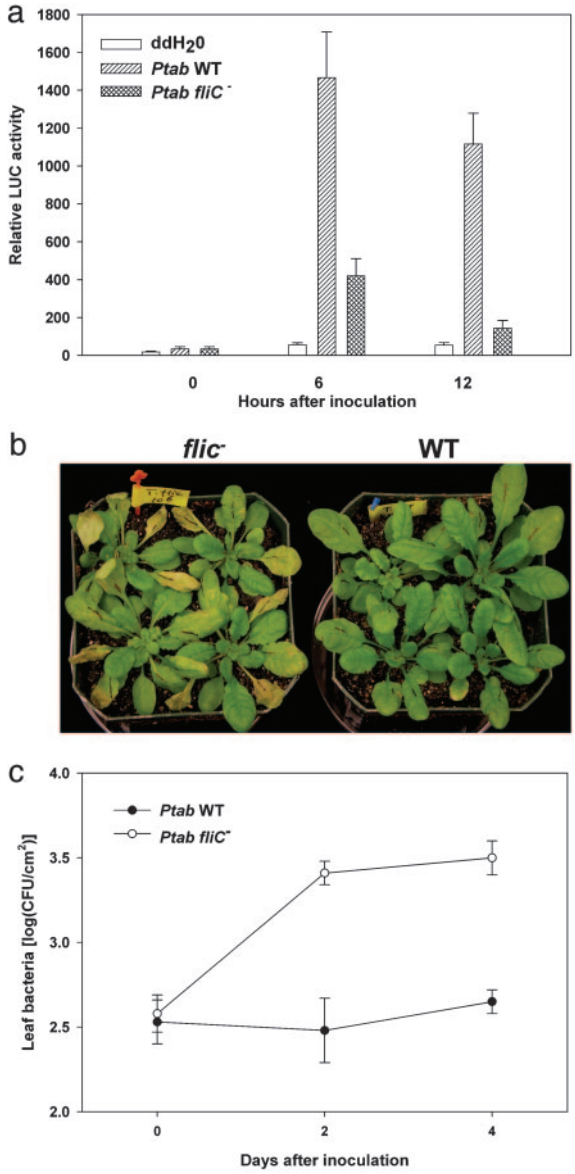

Fig. 2. Flagellin is required for *NHO1* induction and resistance to *Ptab*. (*a*) *NHO1–LUC* activity of plants inoculated with the wild-type and *fliC*$^-$ mutant strains of *Ptab*. (*b*) Disease symptoms of *Arabidopsis* plants (Col-0) 7 days after inoculation with the wild-type (WT) and *fliC*$^-$ mutant strains of *Ptab* (10^6 cfu/ml). (*c*) Bacterial growth of the wild-type (WT) and *fliC*$^-$ mutant *Ptab* strains on *Arabidopsis* plants (Col-0). The experiments were repeated three times with similar results.

killed by kanamycin did not induce *NHO1–LUC* (data not shown). Bacterial growth assay indicated that the wild-type and *fliC*$^-$ mutant of DC3000 grew similarly when infiltrated into *Arabidopsis* plants (Fig. 3*b*). The two strains also caused indistinguishable disease symptoms (data not shown). These results demonstrate that, like *Ptab*, DC3000 flagellin is fully capable of inducing *NHO1*. However, unlike *Ptab*, the response to DC3000 flagellin is abrogated and does not result in resistance in the plant.

TTSS Is Essential for DC3000 to Suppress *NHO1*. The lack of sustained *NHO1–LUC* induction by DC3000 flagellin is consistent with our hypothesis that this bacterium actively suppresses the *NHO1*-mediated nonhost resistance (19). Therefore, a role of DC3000 virulence/pathogenicity genes in the active suppression of *NHO1* was tested. Fig. 4*a* shows that DC3000 strains lacking the TTSS structure genes *hrpA* and *hrcC* induced much greater *NHO1–LUC* expression compared with the wild-type DC3000, indicating that TTSS is largely responsible for the suppression. The DC3000 mutant lacking the regulatory gene *hrpL* gave an even stronger induction than did *hrpA*$^-$ and *hrcC*$^-$ mutants (Fig. 4*a*). The strength and kinetics of the *hrpL*$^-$ mutant-induced *NHO1–LUC* expression resemble those of *Pph* (Figs. 4*b* and 1*b*). *hrpL* encodes a sigma factor that regulates both TTSS and coronatine biosynthetic genes through the *hrp* box (29). These results demonstrate that TTSS is essential for DC3000 to suppress the *NHO1* expression.

Type III Effectors Suppress *NHO1* Expression. The hypothesis that type III effectors suppress *NHO1* expression was systematically tested by using a protoplast-based transient assay. Protoplasts were isolated from plants carrying the *NHO1–LUC* reporter and transfected with constructs carrying DC3000 effector genes under the control of the cauliflower mosaic virus 35S promoter. A total of 19 effectors were tested (Table 1; www.pseudomonas-syringae.org). Most of these

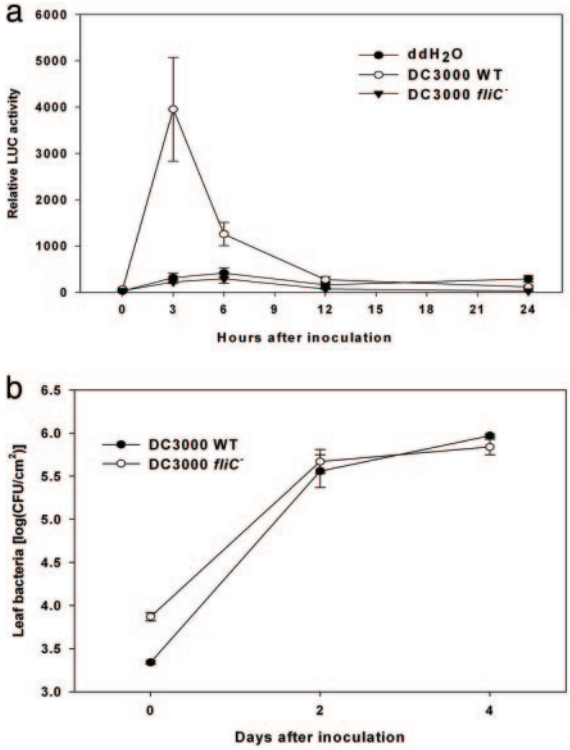

Fig. 3. DC3000 flagellin transiently induces *NHO1* and fails to confer disease resistance. (*a*) *NHO1–LUC* expression in plants inoculated with water, the wild-type (WT) or *fliC*$^{-}$ mutant DC3000 strains at the indicated hours after inoculation. (*b*) Bacterial growth assay of Col-0 plants infiltrated with the wild-type (WT) or *fliC*$^{-}$ mutant strains of DC3000. The experiments were repeated three times with similar results.

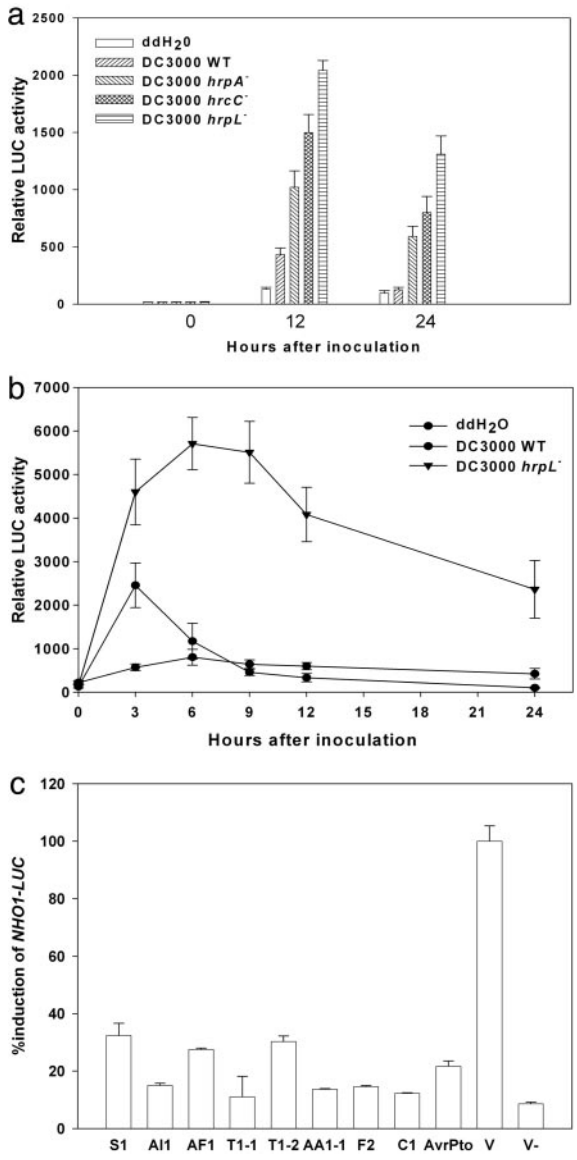

Fig. 4. DC3000 requires type III effectors to suppress *NHO1* expression. (*a*) *NHO1–LUC* plants were inoculated with the wild-type (WT), *hrpA*$^{-}$, *hrcC*$^{-}$, or *hrpL*$^{-}$ mutant DC3000 strains, and relative luciferase activity was measured 0, 12, and 24 h after inoculation. (*b*) Kinetics of *NHO1–LUC* expression in response to the wild-type (WT) and *hrpL*$^{-}$ mutant DC3000 strains. (*c*) Expression of DC3000 effectors blocks flg22-induced *NHO1–LUC* expression. Protoplasts were transfected either with the empty vector (V) or the indicated effector constructs, and relative LUC activity was measured 12 h after addition of flg22. Vector-transfected protoplasts treated with ddH_2O were used as a control for basal *NHO1–LUC* expression (V−). Each data point consists of three replicates. The error bar represents standard error. The experiments were repeated three times with similar results.

effectors were selected because their function in virulence had not been reported previously. For control, protoplasts were transfected with an empty vector. The transfected protoplasts were subsequently induced with flg22$^{P.s.tabaci}$. As seen in Fig. 4*c*, flg22 $^{P.s.tabaci}$ induced *NHO1–LUC* in protoplasts transfected with empty vector compared to uninduced protoplasts, recapitulating the *NHO1–LUC* induction observed in intact leaves. Transfection of nine effector genes, *hopS1*, *hopAI1*, *hopAF1*, *hopT1-1*, *hopT1-2*, *hopAA1-1*, *hopF2*, *hopC1*, and *avrPto*, strongly reduced the flagellin-induced *NHO1* expression in repeated experiments. Among these, *hopAI1*, *hopT1-1*, *hopAA1-1*, *hopF2*, and *hopC1* completely abolished the *NHO1* induction. Other effector genes did not show a consistent effect on *NHO1* induction. These results indicate that almost 50% of the tested DC3000 effectors are functionally redundant and suppress the flagellin-induced *NHO1* expression.

Southern blot analysis was carried out to determine whether any of these nine effector sequences exist in the two nonhost strains used (Fig. 9). Not all of the nine effectors described in this work are unique to DC3000. HopAA1 is encoded by the conserved effector locus that exists in all known *P. syringae* pathovars (30). Southern blot analysis indicated that the *hopT1-1* and *hopAA1* sequences exist in *Ptab*, whereas the *hopAF1*, *hopT1-2*, and *hopAA1* sequences are present in *Pph*. Thus, it appears that the delivery of a few of these effectors by the bacterium is not sufficient for the suppression.

HopAI1 Promotes Parasitism in Plants. To determine whether any of the tested effectors promote virulence, a FLAG-tagged *hopAI1* was introduced into *Arabidopsis* plants as a stable transgene by using an estrodial-inducible system (28). This effector was chosen because it shares 35% identity with the *Salmonella enterica* serovar *typhimurium* VirA, a mouse killing factor (ref. 31 and Fig. 1). A search of the GenBank database indicated that similar proteins also exist in *Salmonella choleraesuis*, *Shigella flexneri*, and *Chromobacterium violaceum.* Fig. 5*a* shows that induced expression of *hopAI1* in a transgenic line exhibited chlorosis, reminiscent of disease symptoms. The expression of *hopAI1* also enhanced the growth of the *hrpL*$^{-}$ mutant bacteria by at least 30-fold (Fig. 5*b*). Similar results were observed in six primary transgenic plants (Fig. 8). These results indicate that the suppression of *NHO1* by HopAI1 is relevant to the virulence function.

The role of *hopAI1* in *NHO1*-suppression was further tested by using a DC3000 mutant strain carrying truncated *hopAI1.* Consis-

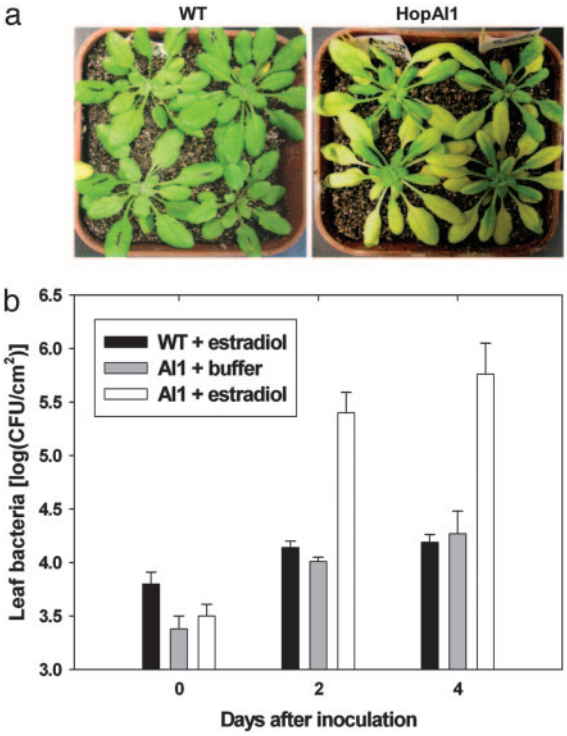

Fig. 5. HopAI1 promotes virulence in plants. (*a*) *hopAI1* expression induces chlorosis. Transgenic *hopAI1-FLAG* (line 2) and wild-type (WT) plants were sprayed with 50 μM estradiol and photographed 5 days later. (*b*) *hopAI1* expression enhances bacterial growth in plants. Transgenic *hopAI1-FLAG* (line 2) and wild-type (WT) plants were sprayed with either buffer or 50 μM estradiol 1 day before inoculation with the *hrpL*$^-$ mutant. Bacteria population in the leaf was determined at the indicated times. Error bars indicate standard error.

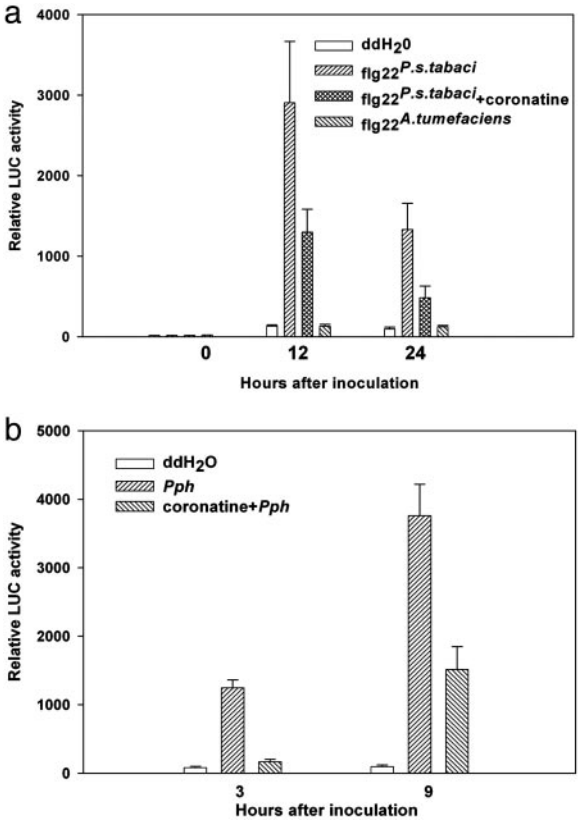

Fig. 6. Coronatine partially suppresses *NHO1* expression. (*a*) Purified coronatine inhibits the flagellin-induced *NHO1–LUC* expression. *NHO1–LUC* activity of plants infiltrated with water, 1 μM flg22$^{P.s.tabaci}$ alone, 1 μM flg22$^{P.s.tabaci}$ plus 200 ng/ml purified coronatine, or 1 μM flg22$^{A.tumefaciens}$. (*b*) Purified coronatine inhibits the *Pph*-induced *NHO1–LUC* expression. *NHO1–LUC* activity of plants inoculated with water, *Pph*, or *Pph* plus 200 ng/ml coronatine (cronatine+*Pph*). The experiments were repeated twice with similar results.

tent with a redundant role of multiple effectors in *NHO1* suppression, the *hopAI1* mutation did not produce a measurable effect on *NHO1–LUC* suppression (data not shown).

Coronatine Partially Suppresses *NHO1* Expression. Previous work suggested that both TTSS and the phytotoxin coronatine modulate the expression of a similar set of plant genes (21, 30). This suggestion prompted us to test whether coronatine also contributes to the observed suppression of *NHO1*. Fig. 6 *a* and *b* show that coinfiltration of purified coronatine diminished the *NHO1–LUC* expression induced by flg22$^{P.s.tabaci}$ or *Pph*. However, a DC3000 mutant that is blocked in the synthesis of coronatine was only marginally compromised in *NHO1–LUC* suppression (data not shown). Together, these results suggest that coronatine plays a minor role in *NHO1* suppression. A role of coronatine and the requirement of COI1 in *NHO1* suppression (20) indicate that jasmonate signaling may play a role in *NHO1* suppression. Consistent with this possibility, exogenous application of methyl jasmonate partially suppressed the *Pph*-induced *NHO1–LUC* expression (data not shown).

Discussion

The molecular basis of nonhost resistance is poorly understood. It is speculated that PAMP-induced innate immunity plays an important role in the species level resistance, but direct evidence is lacking (4). The results presented here show that flg22, a known PAMP, mimics nonhost bacteria and induces the expression of *NHO1*. In contrast, the inactive peptide flg22$^{A.tumefaciens}$ is unable to induce *NHO1*. Thus, the induced expression of the nonhost resistance gene *NHO1* is a typical PAMP-mediated innate immune response.

Recent results showed that *Pseudomonas* bacteria carry at least two additional PAMPs, a cold-shock protein and elongation factor-TU, both inducing defense responses in plants (32, 33). The results presented here indicate that flagellin is the primary PAMP in *Ptab* responsible for *NHO1* induction, because the *fliC*$^-$ mutant strain is largely inactive in *NHO1* induction. The induction of *NHO1* is likely of functionally importance, because *Arabidopsis* plants overexpressing *NHO1* display enhanced resistance to DC3000 (20). The *Ptab* strain lacking *fliC* gains partial virulence on wild-type *Arabidopsis* when directly infiltrated into leaves. This strain also displays enhanced virulence on tomato plants (23). It should be noted that the *fliC*$^-$ mutant is not fully pathogenic on *Arabidopsis*. One plausible explanation is that PAMPs other than flagellin also contribute to species level resistance (9). Nevertheless, these results demonstrate that flagellin plays a critical role in eliciting nonhost resistance.

Although nonhost resistance is effective to the vast majority of potential pathogens, it is breached by a small number of pathogens, presumably because the latter has evolved specialized virulence mechanisms that enable them to successfully overcome this resistance. Flagellin is highly conserved among Pseudomonads, including DC3000, which is virulent on *Arabidopsis*. *NHO1–LUC* reporter assay revealed a transient induction by DC3000, and this induction is flagellin-dependent. The induction is quickly suppressed within 6 h after inoculation, and coincides with the *in planta* expression of type III genes in DC3000 (34). We previously hypothesized that DC3000 suppresses *NHO1* by using type III effectors (20). Indeed, the *hrpA*$^-$, *hrcC*$^-$, and *hrpL*$^-$ mutants of DC3000 all induce *NHO1–LUC* to a much greater level than does the wild-type strain.

Most importantly, direct expression of nine DC3000 effector genes in the plant cell or exposure to purified coronatine strongly suppresses the flg22-induced expression of *NHO1–LUC*, providing direct evidence that type III effectors suppress the flagellin-induced immune responses. These observations are consistent with the knowledge that exogenous flagellin only protects *Arabidopsis* plants against DC3000 when applied 1 day before the bacterial inoculation, but ineffective when infiltrated simultaneously with the DC3000 bacterium (9). Together, these results provide strong evidence that a major target for DC3000 is innate immunity that acts at the species level to limit nonhost *Pseudomonas* bacteria. Consistent with the role of DC3000 TTSS in overcoming species level resistance, recent work shows that the DC3000 TTSS actively suppresses and tolerates the production of antimicrobial root exudates that are inhibitory to nonhost bacteria, although which effector(s) does so remains to be determined (35). The ability of a bacterium to overcome the species level resistance may represent a major evolutionary step that enables a *P. syringae* bacterium to colonize on a new host species.

The results presented here indicate that a surprisingly large proportion of the DC3000 effectors possesses the ability to suppress the flagellin-induced *NHO1* expression. Among the nine effectors that suppress *NHO1* expression, at least HopAI1 and AvrPto are capable of promoting nonpathogenic bacterial growth when expressed in plants (36). HopAI1 shares significant homology with virulence proteins of animal bacteria. This finding raises an intriguing possibility that flagellin-induced innate immunity in the host is similarly targeted by diverse pathogenic bacteria. Expression of AvrPto in the plant also suppresses callose deposition induced by the *hrcC* mutant bacteria (36). Because callose deposition can be induced by flagellin (5), AvrPto might suppress cell wall defense and *NHO1* expression through a common step required for flagellin signaling. A recent report shows that AvrRpt2 and AvrRpm1 can suppress flagellin-induced callose deposition when directly expressed in plants (37). These observations reinforce the notion that flagellin-induced defenses are targeted by diverse effectors, although they do not appear to share a conserved biochemical function.

A large number of *P. syringae* effectors have been shown to target various host defenses including callose deposition, defense gene expression and cell death induced by gene-for-gene interaction or nonhost interactions (17). Often, the defense suppression by an individual effector is revealed either when the latter is directly expressed at a high level in the plant cell or delivered along with other effectors in the bacterium. It remains to be determined whether these effectors, when individually delivered by *P. syringae*, are sufficient to suppress host defenses. It is possible that a successful defense suppression by a bacterium requires synergistic action of a large set of the bacterium-delivered effectors. For instance, conserved effector locus (CEL), which exists in all *P. syringae*, is required by DC3000 for pathogenicity and suppression of callose deposition in *Arabidopsis* (38). However, the vast majority of *P. syringae* is nonpathogenic on *Arabidopsis*. Thus, the function of CEL-encoded effectors is likely to be assisted by other effectors unique to DC3000. Similarly, several effectors activate COI1-dependent gene expression when delivered by DC3000 but not when delivered by *Pph* (21). DC3000 type III effectors and coronatine act synergistically to modulate the JA signaling in *Arabidopsis* (21, 39). These may explain why some of the nine effector sequences carried by *Ptab* and *Pph* do not appear to suppress the *NHO1* expression. It may be that the suppression of the flagellin-induced expression of *NHO1*, which is known to involve the JA signaling pathway (20), requires a synergistic activity from a large set of these effectors and coronatine that target the JA signaling pathway.

The RPM1-interacting protein RIN4 was shown recently to negatively regulate the flagellin-induced callose deposition (35). RIN4 also interacts with AvrRpt2 and AvrRpm1 (40–42). AvrRpt2 is a cysteine protease that cleaves RIN4, leading to the degradation of RIN4 (43), whereas the AvrRpm1 interaction results in the phosphorylation of RIN4 (40). It is suggested that RIN4 and/or RIN4-associated proteins are manipulated by these two effectors to suppress the flagellin-induced cell wall defense (35). It remains to be shown whether and how the AvrRpt2-mediated degradation of RIN4 leads to the suppression of callose deposition. An important area of future research will be to determine whether a common mechanism is used by various effectors to suppress flagellin-induced defenses.

We thank Sheng-Yang He (Michigan State University, East Lansing), Barbara Kunkel (Washington University, St. Louis), and Yuki Ichinose (Okayama University, Okayama, Japan) for providing bacterial strains Nam-Hai Chua (The Rockefeller University, New York) for the pER8 plasmid; and Frank White and Jyoti Shah for critical reading of the manuscript. J.M.Z. was supported by U.S. Department of Agriculture National Research Initiative Grant 2003-01191 and Chinese Ministry of Science and Technology "863" Grant 2003-AA210080. This paper is a contribution of Kansas Agriculture Experimental Station (05-177-J).

1. Heath, M. C. (1987) *Can. J. Plant Pathol.* **9,** 389–397.
2. Martin, G. B., Bogdanove, A. J. & Sessa, G. (2003) *Annu. Rev. Plant Biol.* **54,** 23–61.
3. Belkhadir, Y., Subramaniam, R. & Dangl, J. L. (2004) *Curr. Opin. Plant Biol.* **7,** 391–399.
4. Nurnberger, T., Brunner, F., Kemmerling, B. & Piater, L. (2004) *Immunol. Rev.* **198,** 249–266.
5. Felix, G., Duran, J. D., Volko, S. & Boller, T. (1999) *Plant J.* **18,** 265–276.
6. Gomez-Gomez, L., Felix, G. & Boller, T. (1999) *Plant J.* **18,** 277–284.
7. Gomez-Gomez, L. & Boller, T. (2000) *Mol. Cell* **5,** 1003–1011.
8. Asai, T., Tena, G., Plotnikova, J., Willmann, M. R., Chiu, W.-L., Gomez-Gomez, L., Boller, T., Ausubel, F. M. & Sheen, J. (2002) *Nature* **415,** 977–983.
9. Zipfel, C., Robatzek, S., Navarro, L., Oakeley, E. J., Jones, J. D., Felix, G. & Boller, T. (2004) *Nature* **428,** 764–767.
10. Alfano, J. R. & Collmer, A. (2004) *Annu. Rev. Phytopathol.* **42,** 385–414.
11. Chen, Z., Kloek, A. P., Boch, J., Katagiri, F. & Kunkel, B. N. (2000) *Mol. Plant–Microbe Interact.* **13,** 1312–1321.
12. Ritter, C. & Dangl, J. L. (1995) *Mol. Plant–Microbe Interact.* **8,** 444–453.
13. Jackson, R. W., Athanassopoulos, E., Tsiamis, G., Mansfield, J. W., Sesma, A., Arnold, D. L., Gibbon, M. J., Murillo, J., Taylor, J. D. & Vivian, A. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 10875–10880.
14. Tsiamis, G., Mansfield, J. W., Hockenhull, R., Jackson, R. W., Sesma, A., Athanassopoulos, E., Bennett, M. A., Stevens, C., Vivian, A., Taylor, J. D. & Murillo, J. (2000) *EMBO J.* **19,** 3204–3214.
15. Shan, L., He, P., Zhou, J. M. & Tang, X. (2000) *Mol. Plant–Microbe Interact.* **13,** 592–598.
16. Abramovitch, R. B., Kim, Y. J., Chen, S., Dickman, M. B. & Martin, G. B. (2003) *EMBO J.* **22,** 60–69.
17. Abramovitch, R. B. & Martin, G. B. (2004) *Curr. Opin. Plant Biol.* **7,** 356–364.
18. Bender, C. L., Alarcon-Chaidez, F. & Gross, D. C. (1999) *Microbiol. Mol. Biol. Rev.* **63,** 266–292.
19. Lu, M., Tang, X. & Zhou, J.-M. (2001) *Plant Cell* **13,** 437–447.
20. Kang, L., Li, J., Zhao, T., Xiao, F., Tang, X., Thilmony, R., He, S. & Zhou, J. M. (2003) *Proc. Natl. Acad. Sci. USA* **100,** 3519–3524.
21. He, P., Chintamanani, S., Chen, Z., Zhu, L., Kunkel, B. N., Alfano, J. R., Tang, X. & Zhou, J. M. (2004) *Plant J.* **37,** 589–602.
22. Clough, S. J. & Bent, A. F. (1998) *Plant J.* **16,** 735–743.
23. Shimizu, R., Taguchi, F., Marutani, M., Mukaihara, T., Inagaki, Y., Toyoda, K., Shiraishi, T. & Ichinose, Y. (2003) *Mol. Genet. Genomics* **269,** 21–30.
24. Zwiesler-Vollick, J., Plovanich-Jones, A. E., Nomura, K., Bandyopadhyay, S., Joardar, V., Kunkel, B. N. & He, S. Y. (2002) *Mol. Microbiol.* **45,** 1207–1218.
25. Roine, E., Wei, W., Yuan, J., Nurmiaho-Lassila, E.-L., Kalkkinen, N., Romantschuk, M. & He, S. Y. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 3459–3464.
26. Yuan, J. & He, S. Y. (1996) *J. Bacteriol.* **178,** 6399–6402.
27. Hu, W., Yuan, J., Jin, Q. L., Hart, P. & He, S. Y. (2001) *Mol. Plant–Microbe Interact.* **14,** 234–241.
28. Zuo, J., Nu, Q. & Chua, N.-H. (2000) *Plant J.* **24,** 265–273.
29. Boch, J., Joardar, V., Gao, L., Robertson, T. L., Lim, M. & Kunkel, B. N. (2002) *Mol. Microbiol.* **44,** 73–88.
30. Alfano, J. R., Charkowski, A. O., Deng, W. L., Badel, J. L., Petnicki-Ocwieja, T., van Dijk, K. & Collmer, A. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 4856–4861.
31. Gulig, P. A. & Chiodo, V. A. (1990) *Infect. Immun.* **58,** 2651–2658.
32. Felix, G. & Boller, T. (2003) *J. Biol. Chem.* **278,** 6201–6208.
33. Kunze, G., Zipfel, C., Robatzek, S., Niehaus, K., Boller, T. & Felix, F. (2004) *Plant Cell* **16,** 3496–3507.
34. Xiao, F., Goodwin, S. M., Xiao, Y., Sun, Z., Baker, D., Tang, X., Jenks, M. A. & Zhou, J. M. (2004) *EMBO J.* **23,** 2903–2913.
35. Bais, H. P., Prithiviraj, B., Jha, A. K., Ausubel, F. M. & Vivanco, J. M. (2005) *Nature* **434,** 217–221.
36. Hauck, P., Thilmony, R. & He, S. Y. (2003) *Proc. Natl. Acad. Sci. USA* **100,** 8577–8582.
37. Kim, M. G., Cunha, L. D., McFall, A. J., Belkhadir, Y., DebRoy, S., Dangl, J. L. & Mackey, D. (2005) *Cell* **121,** 749–759.
38. DebRoy, S., Thilmony, R., Kwack, Y. B., Nomura, K. & He, S. Y. (2004) *Proc. Natl. Acad. Sci. USA* **101,** 9927–9932.
39. Zhao, Y., Thilmony, R., Bender, C. L., Schaller, A., He, S. Y. & Howe, G. A. (2003) *Plant J.* **36,** 485–499.
40. Mackey, D., Holt, B. F., Wiig, A. & Dangl, J. L. (2002) *Cell* **108,** 743–754.
41. Mackey, D., Belkhadir, Y., Alonso, J. M., Ecker, J. R. & Dangl, J. L. (2003) *Cell* **112,** 379–389.
42. Axtell, M. J. & Staskawicz, B. J. (2003) *Cell* **112,** 369–377.
43. Day, B., Dahlbeck, D., Huang, J., Chrisholm, S. T., Li., D. & Staskawicz, B. J. (2005) *Plant Cell* **17,** 1292–1305.