***Xanthomonas* Filamentous Hemagglutinin-Like Protein Fha1 Interacts with Pepper Hypersensitive-Induced Reaction Protein CaHIR1 and Functions as a Virulence Factor in Host Plants**

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Submitted 22 July 2013. Accepted 25 July 2013.

Pathogens have evolved a variety of virulence factors to infect host plants successfully. We previously identified the pepper plasma-membrane-resident hypersensitive-induced reaction protein (CaHIR1) as a regulator of plant disease- and immunity-associated cell death. Here, we identified the small filamentous hemagglutinin-like protein (Fha1) of *Xanthomonas campestris* pv. *vesicatoria* as an interacting partner of CaHIR1 using yeast two-hybrid screening. Co-immunoprecipitation and bimolecular fluorescence complementation experiments revealed that Fha1 specifically interacts with CaHIR1 in planta. The endocytic tracker FM4-64 staining showed that the CaHIR1-Fha1 complex localizes in the endocytic vesicle-like structure. The *X. campestris* pv. *vesicatoria* Δ fha1 mutant strain exhibited significantly increased surface adherence but reduced swarming motility. Mutation of *fha1* inhibited the growth of *X. campestris* pv. *vesicatoria* and *X. campestris* pv. *vesicatoria* Δ avrBsT in tomato and pepper leaves, respectively, suggesting that Fha1 acts as a virulence factor in host plants. Transient expression of *fha1* and also infiltration with purified Fha1 proteins induced disease-associated cell death response through the interaction with CaHIR1 and suppressed the expression of *pathogenesis-related* (PR) genes. Silencing of *CaHIR1* in pepper significantly reduced Δ avrBsT growth and Fha1-triggered susceptibility cell death.

Overexpression of *fha1* in *Arabidopsis* retarded plant growth and triggered disease-associated cell death, resulting in altered disease susceptibility. Taken together, these results suggest that the *X. campestris* pv. *vesicatoria* virulence factor Fha1 interacts with CaHIR1, induces susceptibility cell death, and suppresses PR gene expression in host plants.

Disease resistance is genetically controlled by pathogen-derived effector proteins and their cognate resistance (R) proteins of host plants (Bent and Mackey 2007; Chisholm et al. 2006; Flor 1971; Jones and Dangl 2006). In effector-triggered immunity (ETI), R proteins recognize an invading pathogen and trigger resistance responses by directly detecting effector proteins or monitoring effector-induced modification of guard proteins (Jones and Dangl 2006). ETI occurs in a cultivar- and race-specific manner. The recognition of general elicitors, which are known as pathogen-associated molecular patterns (PAMPs), induces PAMP-triggered immunity (PTI) in plants (Altenbach and Robatzek 2007; Bent and Mackey 2007). PAMPs are conserved structures of microbes that are essential for their life. The PAMPs include lipopolysaccharides (LPS) of bacteria, chitins of fungi, and β -glucans of oomycetes, among others (Altenbach and Robatzek 2007; Klarzynski et al. 2000). Unlike ETI, PTI occurs in different cultivars of one or many species. Recent studies show that the innate immune response is mediated by recognition of PAMP molecules by plasma membrane (PM)-resident pattern recognition receptors (PRR) (Altenbach and Robatzek 2007; Zipfel 2008). An extensively studied PTI response is FLAGELLIN SENSITIVE 2 (FLS2) receptor kinase-mediated flagellin recognition, which involves the highly conserved flg22 peptide (Chinchilla et al. 2006; Gómez-Gómez et al. 2001; Robatzek et al. 2006). However, few PRR recognizing PAMPs have been characterized in plants.

In animal systems, several PAMPs, such as glucans, chitin, LPS, and flagellin, are also known to activate PTI (Gordon 2002; Nürnberger et al. 2004). The filamentous hemagglutinin (Fha) protein of the whooping cough agent *Bordetella pertussis* was reported to interact with macrophage complement receptor 3 (CR3) (Ishibashi et al. 1994; Relman et al. 1990). Activation and surface expression of CR3 is important for Fha-mediated phagocytosis and subsequent killing of pathogens (Mobberley-

Sequence data from this article can be found in the EMBL/GenBank data libraries under accession numbers KC466081 (*fha1*), AF053343 (*CaPRI*), AF442388 (*CaDEF1*), AY529867 (*CaHIR1*), Y08804 *Senu4* (*PRI*), NM_001247943 *Tgas* (*DEF*), NM_001247203 *Cevi16* (*POX*), and At4g05320 (*UBQ*).

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Nucleotide sequence data of *XcvFHA1* from this article can be found in the GenBank data libraries under accession number KC466081.

*The e-Xtra logo stands for “electronic extra” and indicates that seven supplementary figures and one supplementary table are published online and that Figures 1, 2, 4, 5, 6, 7, 9, and 10 appear in color online.

Schuman and Weiss 2005; Weigart and Weiss 2000). Furthermore, vaccination with purified Fha proteins confers protection against *B. pertussis* and *B. bronchiseptica* in humans and mice, respectively (Goebel et al. 2009; Rieber et al. 2008). These findings suggest that the Fha protein, similar to other PAMPs, can activate immune responses in animal systems. The Fha proteins of gram-negative bacteria function as an adhesin and are highly conserved in their structure at the species level (Link et al. 2007). The well-known Fha protein of *B. pertussis* FhaB has at least three different binding activities, including carbohydrate binding, heparan sulfate binding, and integrin CR3 binding through its Arg-Gly-Asp (RGD) site (Ishibashi et al. 1994; Relman et al. 1990). These binding activities allow the pathogen to adhere to a variety of cells and extracellular structures in the respiratory epithelium. In particular, interaction of FhaB of *B. pertussis* with the macrophage CR3 implicates Fha in the competitive blockade of CR3-mediated leukocyte signal transduction, the delivery of bacterial toxins to host leukocytes, and bacterial intracellular entry for survival and persistence (Ishibashi et al. 1994; Relman et al. 1990). These findings suggest that the bacterial adhesin Fha not only plays a role in disarming the neutrophil defense mechanism but also can act as target protein of CR3-mediated phagocytosis in animal defense responses.

The role of Fha proteins of plant bacterial pathogens has mainly been studied on the basis of their virulence (Gottig et al. 2009; Guilhabert and Kirkpatrick 2005; Rojas et al. 2002). Two functional homologs of *B. pertussis* FhaB were identified from *Erwinia chrysanthemi* and *Xanthomonas axonopodis* pv. *citri* (Gottig et al. 2009; Rojas et al. 2002). Mutation of FhaB homologs in these pathogens decreased their ability to adhere to plant tissues, leading to reduced virulence. In another study, screening of a random transposon (Tn5) mutant library of *Xylella fastidiosa* revealed that the hemagglutinin adhesin gene *HxfB* functions in bacterial cell-to-cell aggregation, which results in colony formation and biofilm maturation within the xylem vessels of grapevine (Guilhabert and Kirkpatrick 2005). These findings suggest that Fha proteins of plant pathogens function as virulence factors by modulating surface attachment, biofilm formation, and cell-to-cell aggregation during pathogenesis. However, whether these Fha proteins are able to elicit plant defense responses or whether the target proteins of Fha exist in plants remains to be determined.

In our previous study, the *Capsicum annuum* hypersensitive induced reaction 1 (CaHIR1) protein was identified as a positive regulator of pathogen-induced cell death associated with disease and immunity (Choi et al. 2011; Jung and Hwang 2007; Jung et al. 2008). Expression of CaHIR1 induced pathogen-independent callose deposition and hypersensitive-like cell death, accompanied by suppression of endogenous salicylic acid accumulation. Its cellular interacting protein *C. annuum* leucine-rich repeat 1 (CaLRR1) also antagonistically modulated CaHIR1 function in the execution of cell death (Choi et al. 2011; Jung et al. 2004; Jung and Hwang 2007). Interestingly, extracellular localized CaLRR1 interacted with PM-associated CaHIR1 to form membrane microdomains that may function as platforms for membrane transport, membrane trafficking, and cytoskeleton interaction (Browman et al. 2007). To identify pathogen-derived molecular partners recognized by CaHIR1, we screened a *Xanthomonas campestris* pv. *vesicatoria* GAL4 yeast two-hybrid (Y2H) prey library using CaHIR1 as bait. Here, we identified a small Fha-like protein, Fha1, of *X. campestris* pv. *vesicatoria* as an interactor of the plant cell death executor CaHIR1. The Y2H and coimmunoprecipitation experiments revealed that Fha1 specifically interacts with CaHIR1 in vitro and in planta. Functional studies using mutant strains carrying the disrupted allele of the *fha1* open reading

frame (ORF) (*X. campestris* pv. *vesicatoria* $\Delta fha1$) revealed that Fha1 acts as a virulence factor during the colonization of the pathogen in plant tissues. Unexpectedly, the $\Delta fha1$ mutant strain showed increased adherence to plastic and plant leaf surfaces. *Agrobacterium*-mediated transient expression of *fha1* and infiltration of purified Fha1 significantly induced a cell death response, and increased callose deposition and the accumulation of defense-related fluorescent compounds in pepper leaves. Loss-of-function analysis of the $\Delta fha1$ mutant in tomato and pepper plants showed that *fha1* functions as a virulence factor of *X. campestris* pv. *vesicatoria* during the compatible interaction with host plants. Taken together, the results of our study indicate that Fha1 of *X. campestris* pv. *vesicatoria* is recognized by membrane-resident CaHIR1 and is required for disease-associated cell death and defense responses in plants.

RESULTS

CaHIR1 physically interacts with Fha1 in yeast and in planta.

We previously identified CaHIR1 as a positive regulator of cell death associated with disease susceptibility and plant immunity (Choi et al. 2011; Jung and Hwang 2007). The CaHIR1 gene encodes a stomatins, prohibitins, flotillins, and HflK/C domain-containing protein that localizes to the PM in a punctate pattern (Choi et al. 2011). Recently, Qi and associates (2011) showed that *Arabidopsis* HIR proteins are enriched in membrane microdomains in association with RPS2. Therefore, we hypothesized that CaHIR1 proteins may function as platforms for compartmentalization of cellular processes such as membrane transport, protein sorting, and signal transduction during pathogen infection (Browman et al. 2007; Qi and Katagiri 2012). To identify pathogen-derived proteins directly interacting with CaHIR1 and modulating its function, we screened a *X. campestris* pv. *vesicatoria* GAL4 Y2H prey library using CaHIR1 as bait. From approximately 5×10^6 clones, we initially identified part of the *X. campestris* pv. *vesicatoria filamentous hemagglutinin-like protein 1* (*fha1*) gene as one of several putative interactors. To detect the full-length *fha1*, an *X. campestris* pv. *vesicatoria* genomic DNA library was constructed using the SuperCos 1 cosmid vector kit (Stratagene, La Jolla, CA, U.S.A.), followed by polymerase chain reaction (PCR)-based screening using the primers listed in Supplementary Table S1. The expected size of approximately 180 bp of the *fha1* fragment was specifically amplified and sequenced from Cosmid 738 carrying an insert of approximately 40 kb. Sequencing analyses of Cosmid 738 identified the full-length sequence of *fha1* (Supplementary Fig. S1). In a targeted Y2H assay, CaHIR1 physically interacted with the full-length Fha1 in yeast (Fig. 1A). A reciprocal interaction experiment confirmed the interaction between CaHIR1 and Fha1. The interaction between CaHIR1 and Fha1 was quantified by measuring the activity of liquid β -galactosidase using *o*-NPG as a substrate (Supplementary Fig. S2). CaHIR1 and Fha1 did not auto-activate reporter gene expression when fused to the GAL4 activation domain (AD). However, the interaction of CaHIR1 with the full-length Fha1 significantly induced β -galactosidase activity.

To determine the specific interaction between CaHIR1 and Fha1, we performed coimmunoprecipitation experiments (Fig. 1B). The His-tagged recombinant Fha1 was expressed and purified in *Escherichia coli*. Total proteins were then extracted from pepper leaves expressing cMyc-tagged CaHIR1 or empty vector. Protein extracts from pepper leaf tissues were incubated with or without His-tagged Fha1, and subjected to immunoprecipitation with an anti-cMyc antibody. After washing, the eluted protein complex was subjected to immunoblot anal-

ysis using the anti-His antibody. The recombinant Fha1 was able to bind specifically to the plant-expressed CaHIR1 (Fig. 1B).

Interaction and nuclear and cytoplasmic localization of Fha1 and CaHIR1.

In our previous bimolecular fluorescence complementation (BiFC) experiments, CaHIR1 showed a punctate localization in the PM in association with CaLRR1 (Choi et al. 2011; Walter et al. 2004). Similarly, *Arabidopsis* HIR1 showed a punctate distribution pattern in the PM (Qi et al. 2011). To examine the heterodimerization of CaHIR1 with Fha1, a BiFC experiment was performed (Fig. 1C) by fusing the signal peptide sequence from *CaPO2*, which encodes extracellular peroxidase (Choi et al. 2007), to the N-terminal region of the Fha1 protein to translocate plant-expressed Fha1 into the extracellular matrix. More recently, we reported that extracellular CaLRR1 interacts with CaHIR1 localized in the punctate site of the PM and the peripheral region of pepper epidermal cells (Choi et al. 2011). Yellow fluorescence protein (YFP) signals induced by heterodimerization of Fha1 with CaHIR1 were rarely detected in the PM; however, the YFP signal was mainly detected in endocytotic vesicle-like structures of heterogeneous sizes, also known as endosomes (Kang et al. 2010). A fraction of heterodimerization-induced fluorescent signals also co-localized with 4',6-diamidino-2-phenylindole (DAPI)-stained nuclei (Supplementary Fig. S3). However, the YFP signal was not detected in the epidermal cells of *Nicotiana benthamiana* leaves infiltrated with single plasmids or empty vectors (data not shown). To more precisely determine subcellular localization of CaHIR1-Fha1 interactions, the endocytic tracer dye FM4-64 (Bolte et al. 2004) was infiltrated into *N. benthamiana* leaves (Fig. 1C). FM4-64 is rapidly incorporated in PM and diffuses laterally into contiguous membranes. The dye moves into internal cellular membranes only through an active endocytotic process, finally reaching vacuolar membranes (Bolte et al. 2004; Kankanala et al. 2007). Most of the yellow fluorescence induced by the Fha1-CaHIR1 interaction was sequestered in the FM4-64 red fluorescence, indicating that the CaHIR1-Fha1 complex localizes in the endocytic vesicle-like structure (Fig. 1C). However, the YFP signals induced by the Fha1-CaHIR1 interactions was relatively large in size and immobile.

The *fha1* gene encodes an Fha-like protein.

Sequence analysis of *fha1* revealed that the gene contains an ORF encoding a putative 445-amino-acid (aa) protein, with a calculated molecular mass of 45.7 kDa. BLAST analysis indicated that the deduced amino acid sequence of Fha1 contains three conserved Fha motifs involved in bacterial cell aggregation (Gottig et al. 2009; Rojas et al. 2002) (Fig. 2A). The translated Fha1 amino acid sequence showed the highest sequence identity (93%) to the FhaB from *X. axonopodis* pv. *citri*. However, Fha1 lacks the conserved two-partner secretion domain, a highly conserved N-proximal region essential for secretion (Gottig et al. 2009). Despite the lack of the two-partner secretion domain, computational analysis of the putative amino acid sequence predicted that Fha1 may be an outer membrane protein (PSORTb, 99.2%). Fha1 (445 aa) is considerably smaller than FhaB of *X. axonopodis* pv. *citri* (4,753 aa). A phylogenetic tree of Fha1 and its close relatives illustrates its proximity to Fha proteins from diverse bacterial species (Fig. 2B). Fha1 from *X. campestris* pv. *vesicatoria* Bv5-4a falls into a clade that consists of the Fha-like proteins from diverse *Xanthomonas* spp.

Expression of *fha1* in *X. campestris* pv. *vesicatoria*.

RNA gel blot analysis was performed to examine the expression pattern of *fha1* in bacterial cells (Supplementary Fig. S4). *X. campestris* pv. *vesicatoria* Bv5-4a was cultured overnight in

yeast-nutrient (YN) medium at 28°C. Bacterial cells were isolated at 4-h intervals after the lag phase. Expression of *fha1* increased gradually in *X. campestris* pv. *vesicatoria* during the exponential phase, reaching maximum levels during the stationary phase.

Altered cell attachment and swarming motility of *X. campestris* pv. *vesicatoria* Δ *fha1*.

To analyze the biological function of *fha1*, deletion mutants were generated from *X. campestris* pv. *vesicatoria* wild-type (WT) strain Bv5-4a by site-directed mutagenesis (Fig. 3A).

Fig. 1. Pepper CaHIR1 interacts with the *Xanthomonas campestris* pv. *vesicatoria* filamentous hemagglutinin-like protein1 (Fha1) in yeast and in planta. **A**, Interaction of CaHIR1 with Fha1 in a GAL4-based yeast two-hybrid system. Plasmids encoding the fusions to the GAL4 activation domain and the GAL4 DNA-binding domain are indicated by AD and BD, respectively. Combinations of the Simian Vacuolating Virus 40 large T antigen (AD/T) with the murine p53 (BD/p53) fusion constructs and human lamin C (BD/Lam) were included as positive and negative controls, respectively. **B**, Coimmunoprecipitation of CaHIR1 with Fha1. The cMyc-tagged CaHIR1 transgene was transiently expressed in pepper leaves by agroinfiltration. Total proteins extracted from the pepper leaves were incubated with or without His-tagged Fha1 and subjected to immunoprecipitation with anti-cMyc antibody-conjugated beads. Total and immunoprecipitated proteins were hybridized with the indicated antibodies. **C**, Bimolecular fluorescence complementation visualization of the CaHIR1/Fha1 interaction in *Nicotiana benthamiana* leaves. Merged images include yellow fluorescence from yellow fluorescence protein (YFP) complementation by HIR1-Fha1 interaction and red fluorescence from the endocytic tracer dye FM4-64. Bars = 50 μ m.

Analysis of *fhaI* expression in *X. campestris* pv. *vesicatoria* and $\Delta fhaI$ mutant strains by reverse-transcription (RT)-PCR (Fig. 3B) showed that full-length *fhaI* transcripts (*fhaI*) were not present in the deletion mutants. However, transcripts corresponding to the N-terminal region of *fhaI* (*fha*^N), which is

located upstream of the insertion mutation, were more abundant in the $\Delta fhaI$ mutant than in the WT *X. campestris* pv. *vesicatoria* strain. To complement the $\Delta fhaI$ deletion mutant by site-directed mutagenesis, approximately 1 kb upstream of *fhaI* was amplified by PCR, cloned into the pVIK112 vector,

Fig. 2. Amino acid sequence alignment and phylogenetic analysis of filamentous hemagglutinin-like protein1 (Fha1) with other Fha-like proteins. **A**, Amino acid sequence alignment of Fha1 with Fha-like proteins from *Xanthomonas axonopodis* pv. *citri* (Xac1815, accession number NP_642141; Xac1816, accession number NP_642142; Xac1818, accession number NP_642144), *X. campestris* pv. *vesicatoria* (Xcv1861, accession number YP_363592), *Ralstonia solanacearum* (RSc0887, accession number NP_519008), *Burkholderia phyatum* (BphY_7307, accession number YP_001863342), *Pseudomonas syringae* (PSPTO_3229, accession number NP_793014; PSPTO_3210, accession number NP_792996), *Erwinia chrysanthemi* (HecA, accession number AAN38708), *Bordetella petrii* (Bpet0601, accession number YP_001629204), *X. oryzae* (Xoryp_010100020055, accession number ZP_02244872; Xoryp_010100020030, accession number ZP_02244867; Xoryp_010100020065, accession number ZP_02244874), and *Escherichia coli* (accession number AAQ19127). Three filamentous hemagglutinin domains (FH-1, FH-2, and FH-3) are indicated by lines over the sequences. C-proximal regions of Fha1 used for the generation of Fha1-specific peptide antibodies are indicated by a red box. **B**, An unrooted neighbor-joining dendrogram depicting the relationships among 27 representative Fha-like proteins. The scale value of 0.1 represents 0.1 amino acid substitutions per site.

and used for bi-parental mating (Kalogeraki and Winans 1997). Complementation of *fha1* was confirmed by PCR amplification using chromosomal DNA of *X. campestris* pv. *vesicatoria* strains and by RT-PCR (Fig. 3B). Expression of *fha1* in the $\Delta fha1$ (*fha1*) strain was recovered to the WT *X. campestris* pv. *vesicatoria* level, indicating that the *fha1* ORF was fully complemented.

Fha proteins act as a major virulence factor to regulate bacterial adhesion and aggregation in plant and animal pathogens (Gottig et al. 2009; Kajava et al. 2001; Rojas et al. 2002). An in vitro adhesion assay using PVC microtiter well plates was performed to investigate whether the *fha1* mutation reduces bacterial adhesion. The *X. campestris* pv. *vesicatoria*, $\Delta fha1$, and $\Delta fha1$ (*fha1*) strains grown in YN broth were washed three times with phosphate-buffered saline (PBS) buffer (pH 7.0) and incubated in the wells for 6 h. After washing off the planktonic bacterial cells, the remaining attached cells were stained with crystal violet (CV) (Fig. 4A). Solubilization of the CV stain in ethanol allowed an indirect, quantitative measurement of the adherent cell mass in a given well. Unexpectedly, the $\Delta fha1$ strain was more adherent to the surface of well plates than the WT *X. campestris* pv. *vesicatoria* and $\Delta fha1$ (*fha1*) strains, with threefold higher levels of CV stain retained. Consistent with this observation on the artificial plastic surface, $\Delta fha1$ also strongly adhered to the abaxial surface of tomato leaves compared with the WT and complemented strains (Fig. 4B), as shown by the high CV stain retention caused by $\Delta fha1$ adhesion. Next, the effect of the *fha1* mutation on bacterial

motility was examined by swarm plate analysis (Fig. 4C). The $\Delta fha1$ strain showed significantly lower swarming motility on the Silva Buddenhagen (SB) plate than the WT *X. campestris* pv. *vesicatoria* and complemented $\Delta fha1$ (*fha1*) strains (Gottig et al. 2009) (Fig. 4C). Measurement of motility zones after different incubation times showed that the *X. campestris* pv. *vesicatoria* and $\Delta fha1$ (*fha1*) strains moved faster than $\Delta fha1$ (Fig. 4C). Taken together, these results indicate that mutation of *fha1* enhanced the attachment of *X. campestris* pv. *vesicatoria* to various surfaces but reduced its swarming motility.

Reduced virulence

of *X. campestris* pv. *vesicatoria* $\Delta fha1$ in tomato plants.

To investigate the role of *fha1* in bacterial growth in host plants, the growth of the *X. campestris* pv. *vesicatoria*, $\Delta fha1$,

Fig. 3. Generation of *Xanthomonas campestris* pv. *vesicatoria* $\Delta fha1$ and $\Delta fha1$ (*fha1*) mutants from *X. campestris* pv. *vesicatoria* Bv5-4a. **A**, Schematic arrangement of the *fha1* genomic region and generation of the $\Delta fha1$ mutant. The approximately 6-kb pKnockout-G plasmid containing the *fha1* fragment was introduced into the wild-type Bv5-4a by triparental mating. Primer locations and amplified regions of *fha1^N* and *fha1^{Full}* in reverse-transcription polymerase chain reaction (RT-PCR) analyses are shown by black arrowheads and gray boxes, respectively. Sp^r = resistance to spectinomycin and TP = transposase. **B**, RT-PCR analyses of the expression of *fha1* in *X. campestris* pv. *vesicatoria*, $\Delta fha1$, and $\Delta fha1$ (*fha1*) strains. Different regions (*fha1^N* and *fha1^{Full}*) of the *fha1* gene were amplified.

Fig. 4. Increased attachment and reduced swarming motility of the *Xanthomonas campestris* pv. *vesicatoria* $\Delta fha1$ mutant strain compared with *X. campestris* pv. *vesicatoria* and $\Delta fha1$ (*fha1*). **A**, Representative images and crystal violet (CV) staining to visualize the adherence of bacteria to plastic (PVC) surfaces are shown. The adherence of bacteria to the plastic microwells was spectrophotometrically quantified after CV staining. Column 1, wild-type *X. campestris* pv. *vesicatoria*; column 2, $\Delta fha1$; column 3, $\Delta fha1$ (*fha1*). **B**, Representative images and CV staining to visualize the adherence of bacteria to 20 stained spots on the surface of tomato leaves are shown. The adherence of bacteria to the abaxial surface of tomato leaves was spectrophotometrically quantified after CV staining. Column 1, wild-type *X. campestris* pv. *vesicatoria*; column 2, $\Delta fha1$; column 3, $\Delta fha1$ (*fha1*). **C**, Representative images and diameters of the motility zones of swarming bacteria on Silva Buddenhagen plates fortified with 0.7% agar. Data are the means $\pm$ standard deviations from four independent experiments. Different letters indicate significant differences as determined by Fisher's least significant difference test ($P < 0.05$).

Fig. 5. Reduced virulence of the *Xanthomonas campestris* pv. *vesicatoria* $\Delta fha1$ mutant strain compared with *X. campestris* pv. *vesicatoria* and $\Delta fha1$ (*fha1*) in tomato leaves. **A**, Disease symptoms in tomato leaves 5 days after spray inoculation with *X. campestris* pv. *vesicatoria*, $\Delta fha1$, and $\Delta fha1$ (*fha1*) (10^6 CFU ml⁻¹). Localized necrotic lesions induced by $\Delta fha1$ are indicated by red arrowheads. **B**, Reduced disease symptoms of tomato leaves inoculated with $\Delta fha1$. Images were acquired 8 days after syringe infiltration with *X. campestris* pv. *vesicatoria* strains (10^5 CFU ml⁻¹). **C**, Bacterial growth in tomato leaves 0, 1, 3, 5, and 7 days after inoculation with *X. campestris* pv. *vesicatoria* strains (10^5 CFU ml⁻¹). **D**, Quantitative real-time reverse-transcription polymerase chain reaction analysis of the expression of defense response genes in leaves infected with *X. campestris* pv. *vesicatoria* strains. Expression values are normalized to the expression of tomato *ACTIN*. **C** and **D**, Data are the means $\pm$ standard deviations from three independent experiments. Different letters indicate significant differences as determined by Fisher's least significant difference test ($P < 0.05$).

and $\Delta fha1$ (*fha1*) strains was examined in tomato leaves at various time intervals after inoculation with *X. campestris* pv. *vesicatoria* (Fig. 5). The WT *X. campestris* pv. *vesicatoria* (strain Bv5-4a carrying *avrBsT*) used in this study elicited the hypersensitive response (HR) in pepper leaves, whereas it triggered a susceptible disease response in tomato leaves (Kim et al. 2010). Tomato leaves spray inoculated with the $\Delta fha1$ mutant showed localized necrotic spots but spreading chlorotic symptoms were not detected (Fig. 5A). By contrast, severe chlorotic disease symptoms developed on the total surface of tomato leaves 5 days after spray inoculation with *X. campestris* pv. *vesicatoria* and $\Delta fha1$ (*fha1*) strains (10^6 CFU ml^{-1}).

Similar to the spray inoculation results (Fig. 5A), disease severity was lower in tomato leaves syringe infiltrated with $\Delta fha1$ than in those treated with *X. campestris* pv. *vesicatoria* and $\Delta fha1$ (*fha1*) (Fig. 5B). Growth of the $\Delta fha1$ mutant strain was significantly inhibited during infection compared with the growth of the *X. campestris* pv. *vesicatoria* and $\Delta fha1$ (*fha1*) strains (Fig. 5C). At 3 to 7 days after infiltration, the growth of $\Delta fha1$ showed a more than fivefold reduction compared with that of the *X. campestris* pv. *vesicatoria* strain in infected tomato leaves. As expected, the $\Delta fha1$ (*fha1*) strain exhibited a growth pattern similar to that of the *X. campestris* pv. *vesicatoria* strain in infected tomato leaves, suggesting that the reduced virulence of $\Delta fha1$ is caused by the deletion of *fha1*.

We next investigated whether the *fha1* mutation affects the expression of *pathogenesis-related* (*PR*) genes in tomato leaves (Fig. 5D). The *PR* genes, including *Senu4* (*PR1*) (Cunnac et al. 2007), *Tgas118* (defensin) (van den Heuvel et al. 2001), and

Cevi16 (peroxidase) (Gadea et al. 1996), were significantly downregulated in tomato leaves infected with $\Delta fha1$ compared with *X. campestris* pv. *vesicatoria* and $\Delta fha1$ (*fha1*) infections. These data indicate that *fha1* functions as a positive regulator of *PR* gene expression in tomato plants during *X. campestris* pv. *vesicatoria* infection.

Reduced virulence of *X. campestris* pv. *vesicatoria* $\Delta avrBsT/\Delta fha1$ in pepper plants.

We investigated whether the *fha1* mutation affects the *AvrBsT*-triggered HR in pepper leaves infected with *X. campestris* pv. *vesicatoria* Bv5-4a (Fig. 6A and B). Inoculation with $\Delta fha1$ or $\Delta fha1$ (*fha1*) induced a similar HR as *X. campestris* pv. *vesicatoria* infection on pepper leaves (Fig. 6A). Bacterial growth in pepper leaves during infection for 7 days was not significantly different between $\Delta fha1$ and *X. campestris* pv. *vesicatoria* and $\Delta fha1$ (*fha1*) (Fig. 6B). These findings indicate that *fha1* expression may not inhibit the ETI in pepper.

Recently, we reported that mutation of *avrBsT* rendered the avirulent *X. campestris* pv. *vesicatoria* Bv5-4a virulent in pepper plants (Kim et al. 2010). Therefore, we generated the *X. campestris* pv. *vesicatoria* $\Delta avrBsT/\Delta fha1$ mutant in the background of *X. campestris* pv. *vesicatoria* $\Delta avrBsT$. As previously reported, $\Delta avrBsT$ showed a higher level of virulence in pepper plants than $\Delta avrBsT/\Delta fha1$ 7 and 14 days after inoculation (Fig. 6C). $\Delta avrBsT$ infection induced severe disease symptoms in pepper leaves. Growth of the $\Delta avrBsT/\Delta fha1$ mutant in pepper leaves was significantly lower than that of $\Delta avrBsT$ (Fig. 6D). These results indicate that *fha1* functions as a virulence factor of *X. campestris* pv. *vesicatoria* during the compatible interaction with host plants.

Fig. 6. Reduced virulence of *Xanthomonas campestris* pv. *vesicatoria* $\Delta avrBsT/\Delta fha1$ compared with $\Delta fha1$ (*fha1*) in pepper leaves. **A**, Hypersensitive responses in pepper leaves 1 day after inoculation with *X. campestris* pv. *vesicatoria*, $\Delta fha1$, and $\Delta fha1$ (*fha1*) (10^8 CFU ml^{-1}). **B**, Bacterial growth in pepper leaves at different time points after syringe infiltration with *X. campestris* pv. *vesicatoria* strains (10^5 CFU ml^{-1}). **C**, Reduced virulence of $\Delta avrBsT/\Delta fha1$ compared with $\Delta avrBsT$. Images were acquired 7 and 14 days after syringe infiltration of pepper leaves with *X. campestris* pv. *vesicatoria* (10^5 CFU ml^{-1}). **D**, Bacterial growth in pepper leaves at different time points after syringe infiltration with *X. campestris* pv. *vesicatoria* (10^5 CFU ml^{-1}). **B** and **D**, Data are the means $\pm$ standard deviations from three independent experiments. Different letters indicate significant differences as determined by Fisher's least significant difference test ($P < 0.05$). Asterisks indicate significant differences between $\Delta avrBsT$ and $\Delta avrBsT/\Delta fha1$ (Student's *t* test, $P < 0.05$).

Induction of the cell death response in pepper leaves by *Fha1*.

To investigate the in planta function of *Fha1*, we expressed *fha1* in pepper leaves by agroinfiltration. Transient overexpression of *fha1* resulted in cell death around the infiltration sites of pepper leaves (Fig. 7A). By contrast, leaves infiltrated with *Agrobacterium* carrying the empty-vector control (35S:00) did not show cell death symptoms. UV illumination of pepper leaves revealed the accumulation of cell-death-related fluorescent pigments in and around *fha1*-expressing regions 3 and 5 days after agroinfiltration. Only mild chlorotic symptoms were observed in empty-vector-expressing pepper leaves under UV light. The induction of cell death was supported by transient expression of *fha1*, which led to significantly enhanced callose deposition in pepper leaves 3 days after agroinfiltration, indicating that expression of *fha1* triggers cell death responses in pepper leaves (Fig. 7B).

Co-expression of *CaHIR1* with *Fha1* enhances *X. campestris* pv. *vesicatoria* growth and disease susceptibility in pepper leaves.

The interaction of *Fha1* with *CaHIR1* in planta prompted us to investigate whether *CaHIR1* expression is required for virulence and cell-death-inducing activities of *fha1*. $\Delta avrBsT$ and $\Delta avrBsT/\Delta fha1$ strains were used to infect empty-vector control (*Tobacco rattle virus* [TRV]:00) and *CaHIR1*-silenced (TRV:*CaHIR1*) pepper plants. RT-PCR analysis revealed that *CaHIR1* expression was significantly reduced relative to empty-vector control plants (Fig. 8A). This indicates that *CaHIR1* was effectively silenced in pepper plants. *CaHIR1* silencing significantly compromised $\Delta avrBsT$ growth in pepper leaves 3 and 5 days after inoculation (Fig. 8B). However, during $\Delta avrBsT$ /

Fig. 8. **A**, Reverse-transcription polymerase chain reaction analysis of *CaHIR1* expression in empty-vector control (TRV:00) and *CaHIR1*-silenced (TRV:*CaHIR1*) pepper leaves 20 h after inoculation with *Xanthomonas campestris* pv. *vesicatoria* $\Delta avrBsT$ and $\Delta avrBsT/\Delta fha1$ strains (5×10^5 CFU ml⁻¹). **B**, Bacterial growth in empty-vector control and *CaHIR1*-silenced pepper leaves 0, 3, and 5 days after inoculation (dai) with $\Delta avrBsT$ and $\Delta avrBsT/\Delta fha1$ strains (5×10^5 CFU ml⁻¹). Data are the means $\pm$ standard deviations from four independent experiments. Different letters indicate significant differences as determined by Fisher's least significant difference test ($P < 0.05$).

Fig. 7. Induction of cell death responses in pepper leaves by *Agrobacterium*-mediated transient expression of *fha1*. **A**, Accumulation of cell death-related fluorescent pigments in pepper leaves infiltrated with *Agrobacterium tumefaciens* carrying the empty vector pBIN35S (35S:00) or the pBIN35S:*fha1* construct (35S:*fha1*). Visible = a visible symptom on pepper leaves 3 and 5 days after agroinfiltration and UV = UV-exposure of pepper leaves to visualize the deposition of fluorescent pigments and chlorosis. **B**, Aniline blue staining of pepper leaves 3 days after infiltration with *A. tumefaciens* carrying the empty vector pBIN35S (35S:00) or the pBIN35S:*fha1* construct (35S:*fha1*). Agroexpression of *fha1* enhanced callose deposition in pepper leaves. Average numbers of callose foci are shown at the bottom. Data are the means $\pm$ standard deviations from four independent experiments. Different letters indicate significant differences as determined by Fisher's least significant difference test ($P < 0.05$).

Δ *fha1* infection, *CaHIR1* silencing did not significantly reduce bacterial growth in *CaHIR1*-silenced plants, similar to that in the empty-vector control plants. Together, these results indicate that co-expression of *CaHIR1* with *Fha1* enhances *X. campestris* pv. *vesicatoria* growth and also disease susceptibility in pepper leaves.

***CaHIR1* expression enhances induction of disease-associated cell death response by *Fha1* application.**

We next overexpressed and purified His-tagged *Fha1* proteins in *E. coli* (Fig. 9A, left panel). *Fha1* proteins were specifically detected by rabbit antibodies raised against the *Fha1*-specific peptide TAGKWGPDNDKG (Fig. 9A, right panel). The purified recombinant *Fha1* protein was infiltrated into pepper leaves, and the activation of cell death and callose deposition were monitored by trypan blue and aniline blue staining (Supplementary Fig. S5A). Mock-infiltrated leaves did not show cell death induction or callose deposition. However, infiltration with 0.2 μ M *Fha1* significantly increased callose deposition but not cell death induction in pepper leaves. Notably, increasing the concentration of *Fha1* (2 μ M) significantly induced callose deposition as well as cell death. To determine whether the *CaHIR1* is required for *Fha1* to induce callose deposition and cell death, empty-vector- or *CaHIR1*-silenced pepper leaves were infiltrated with 2 μ M *Fha1* (Fig. 9B and C). When analyzed by trypan blue and aniline blue staining, *CaHIR1* silencing significantly reduced cell death and callose deposition induced by *Fha1* application in pepper leaves, respectively (Fig. 9B and C). Together, these results suggest that *Fha1* application induces disease-associated cell death through the interac-

tion with *CaHIR1*, thereby enhancing the virulence of *X. campestris* pv. *vesicatoria*.

Analysis of the transcript levels of *PR* genes, including *CaPR1* and *CaDEF1*, in *Fha1*-infiltrated pepper leaves showed that *CaPR1* and *CaDEF1* expressions were significantly downregulated in pepper leaves 24 h after infiltration with different concentrations of *Fha1*. Higher concentrations of *Fha1* had a greater impact on suppressing *CaPR1* expression. To determine whether *CaHIR1* is required for downregulation of *CaPR1* and *CaDEF1* expression by *Fha1*, we infiltrated *Fha1* into empty-vector- or *CaHIR1*-silenced pepper leaves and monitored *CaPR1* and *CaDEF1* expression. Infiltration with 2 μ M *Fha1* significantly induced a high level of *CaHIR1* expression, and reduced *CaPR1* and *CaDEF1* expression, in the empty-vector control (TRV:00) plants (Fig. 9D). Notably, *CaHIR1* silencing did not affect reduction of *CaPR1* and *CaDEF1* expression by exogenous *Fha1* application. This suggests that *Fha1* may downregulate *CaPR1* and *CaDEF1* expression independently of *CaHIR1* upregulation, leading to the enhanced disease-associated cell death.

Enhanced cell death and altered disease susceptibility of *fha1*-overexpressing transgenic *Arabidopsis*.

Because pepper plants are difficult to transform, we generated *fha1*-overexpressing (OX) transgenic *Arabidopsis* plants to examine the biological function of *fha1* in plants. Constitutive expression of the *fha1* transgene was detected in the *fha1*-OX lines 2, 3, and 4 (Supplementary Fig. S6), which were selected for further experiments. The *fha1*-OX transgenic *Arabidopsis* plants exhibited retarded growth (Fig. 10A) and consti-

Fig. 9. Induction of susceptibility cell death response in the empty vector (TRV:00) and *CaHIR1*-silenced (TRV:*CaHIR1*) pepper leaves by infiltration with purified *Fha1* protein. **A**, Purification and protein gel blot analyses of *Fha1*. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of the purified His-tagged *Fha1* protein (left panel). Gel lanes are as follows: lane 1, soluble crude *Escherichia coli*/*Fha1* extract, noninduced; lane 2, soluble crude *E. coli*/*Fha1* extract, as induced with 1 mM isopropyl- β -D-thio-galactoside; lane 3, recombinant His-*Fha1* fusion protein purified on a Ni-NTA column. Protein gel blot analysis of His-tagged *Fha1* protein using rabbit polyclonal antibodies against *Fha1* (right panel). **B**, Trypan blue and aniline blue staining of pepper leaves 1 day after infiltration with purified *Fha1* protein. Bars = 200 μ m. **C**, Quantification of cell death and callose deposition in leaf tissues by trypan blue and aniline blue staining 24 h after infiltration with purified *Fha1* protein, respectively. **D**, Quantitative reverse-transcription polymerase chain reaction analyses of the expression of *CaHIR1*, *CaPR1* (PR-1), and *CaDEF1* (defensin) in pepper leaves 24 h after infiltration with *Fha1* protein. Expression values are normalized to the expression of pepper 18S rRNA. **C** and **D**, Data are the means $\pm$ standard deviations from four independent experiments. Asterisks indicate significant differences between the empty-vector control (TRV:00) and *CaHIR1*-silenced (TRV:*CaHIR1*) plants (Student's *t* test, *P* < 0.05).

tutive cell death phenotypes as observed by trypan blue staining (Fig. 10B). Cell death phenotypes were mainly observed in the marginal region of the transgenic *Arabidopsis* leaves. Infection with virulent *Pseudomonas syringae* pv. *tomato* DC3000 distinctly induced disease-associated cell death in the *fha1*-OX transgenic leaves compared with the WT plants (Fig. 10C). Consistent with the enhanced disease-associated cell death symptoms, *fha1*-OX transgenic plants showed significantly higher bacterial growth 3 days after inoculation than WT plants (Fig. 10D).

DISCUSSION

Pathogens have evolved various virulence factors that overcome plant surveillance systems by suppressing basal resistance. However, the host proteins targeted by pathogen virulence factors are largely unknown. Recognition of pathogen-derived general elicitors or PAMPs triggers the activation of a variety of downstream defense signaling pathways (Altenbach and Robatzek, 2007; Chinchilla et al. 2006; Kunze et al. 2004). Previously, the pepper PM-resident CaHIR1 protein was identified as a positive regulator of pathogen-induced cell death associated with plant immunity and disease but as a negative regulator of *PR* gene expression (Choi et al. 2011; Jung and Hwang 2007; Jung et al. 2008). CaHIR1 accumulates in pepper leaves during the compatible and incompatible interactions of pepper plants with virulent strain Ds1 and avirulent strain Bv5-4a of *X. campestris* pv. *vesicatoria*, respectively; however, the accumulation of CaHIR1 is stronger and more prolonged in the incompatible interaction. Recently, we identified *X. campestris* pv. *vesicatoria* Bv5-4a harboring the effector protein AvrBsT, which is secreted into plant cells and induces HR-like cell death in pepper plants but suppresses basal defense in tomato plants (Kim et al. 2010). Furthermore, microdomain formation by direct interaction of HIR1 with its cellular partner LRR1 or RPS2 in pepper and *Arabidopsis*, respectively, implicates the role of HIR proteins in the perception of pathogen-derived signaling molecules and in the activation of downstream defense signaling (Choi et al. 2011; Qi et al. 2011). To better understand the CaHIR1-mediated defense signaling, we performed Y2H screening and identified a small Fha-like protein of *X. campestris* pv. *vesicatoria* (Fha1) as an interactor of CaHIR1.

The surface-exposed Fha of gram-negative bacteria is known as one of the most important adhesin proteins, along with fimbriae (Locht et al. 2001). In addition to its importance for bacterial adherence, the Fha protein has been used as an antigen to immunize humans against whooping cough caused by *B. pertussis* (Goebel et al. 2009; Rieber et al. 2008). These findings suggest that Fha acts both as a virulence factor by facilitating bacterial adherence and as an immunogen perceived by the mammalian immune system. Similar to their activity in animal systems, bacterial Fha proteins may play an important role in the adherence of bacteria to plant surfaces and their aggregation in plant tissues (Gottig et al. 2009; Guilhabert and Kirkpatrick 2005; Rojas et al. 2002). However, their target or receptor proteins have not been identified in plants. In the present study, Y2H, coimmunoprecipitation, and BiFC experiments showed that Fha1 specifically interacts with CaHIR1 in vitro and in planta. These findings suggest that CaHIR1 directly recognizes Fha1 during *X. campestris* pv. *vesicatoria* infection. Previously, we showed that PM-localized CaHIR1 binds to the extracellular CaLRR1 in yeast and in planta, suggesting the capability of CaHIR1 to interact with the extracellular proteins (Choi et al. 2011). Computational analysis of the subcellular localization using the PSORTb program predicted that Fha1 may be present in the bacterial outer membrane. Therefore, surface-exposed

Fha1 of *X. campestris* pv. *vesicatoria* may be able to interact with CaHIR1 during infection. However, lack of the conserved two-partner secretion domain in Fha1 raises the possibility that it could reside in the bacterial cytoplasm. Plants and animals have evolved recognition systems not only for microbial surface-exposed elicitors but also for cytoplasmic molecular structures that are essential for the architecture and function of microbial cells (Felix and Boller 2003; Zipfel and Felix 2005). However, it is largely unknown how and where plant or animal cells recognize such pathogen-derived hidden molecules, although lytic enzymes of the hosts and phagocytosis appear to play important roles in releasing these hidden or embedded molecules from microbes (Zipfel and Felix 2005). In any case, it would be interesting to test whether the CaLRR1 or CaHIR1 heterodimer has a high affinity with Fha1. In our previous BiFC analyses, most of the fluorescent signals induced by heterodimerization of CaLRR1 with CaHIR1 were located in the punctate site of the PM and the peripheral region of pepper epidermal cells (Choi et al. 2011). Interestingly, in our present study, YFP signals induced by heterodimerization of Fha1 with CaHIR1 were rarely detected in the PM. However, the co-localization of YFP signal with the endocytic tracer dye FM4-64 revealed that the Fha1-CaHIR1 complex is located in endocytic vesicle-like structures of heterogeneous sizes, known as endosomes (Kang et al. 2010). A fraction of heterodimerization-induced fluorescent signals also was found around the DAPI-stained nuclei. Notably, *Arabidopsis* PM-localized PRR FLS2 was reported to be internalized into the intracellular mobile vesicles within 30 min after stimulation with the flagellin epitope flg22, followed by its

Fig. 10. Increased cell death and disease susceptibility of *fha1*-overexpressing (OX) transgenic *Arabidopsis* plants (numbers 2, 3, and 4) in response to *Pseudomonas syringae* pv. *tomato* DC3000 infection. **A**, Retarded growth of transgenic plants grown on soil for 4 weeks. Visible cell death phenotypes in the *fha1*-OX *Arabidopsis* leaves are indicated by white arrows. **B**, Trypan blue staining of pathogen-independent dead cells in *fha1*-OX *Arabidopsis* leaves. Insets (right panel) show an enlargement of the red-colored boxed areas (left panel). **C**, Leaves of *fha1*-OX *Arabidopsis* plants showed increased disease symptoms 8 days after inoculation with virulent DC3000. **D**, Increased bacterial growth in *fha1*-OX transgenic *Arabidopsis*. Bacterial growth was measured 0 and 3 days after inoculation with virulent DC3000 (10^5 CFU ml⁻¹). Data represent the means $\pm$ standard deviations from three independent experiments. Asterisks indicate significant differences between wild-type (WT) and each of the *fha1*-OX lines (Student's *t* test, $P < 0.05$).

degradation (Robatzek et al. 2006). These findings raise the possibility that Fha1 may also be able to induce internalization of CaHIR1 after their recognition. In *Arabidopsis*, the PM-localized PRR FLS2 and elongation factor Tu (EF-Tu) receptor directly bind flg22 and EF-Tu, respectively, and elicit PTI, which is accompanied by callose deposition and cell death (Chinchilla et al. 2006; Gao et al. 2009; Göhre et al. 2008; Zipfel 2008; Zipfel et al. 2006). Similar to the morphologically distinct phenotype in *Arabidopsis*, *Agrobacterium*-mediated transient expression of *fha1* and infiltration of purified Fha1 also strongly induced susceptibility cell death, accumulation of defense-related fluorescent compounds, and callose deposition in pepper leaves. Similarly, transient expression of *CaHIR1* also induced strong callose deposition in pepper leaves (Choi et al. 2011). These findings strongly suggest that CaHIR1 directly recognizes the *X. campestris* pv. *vesicatoria* virulence factor Fha1 to activate CaHIR1-mediated susceptibility cell death responses in pepper plants, leading to the hypothesis that the recognition of Fha1 by CaHIR1 triggers disease-associated cell death response in pepper plants during *X. campestris* pv. *vesicatoria* infection. Interestingly, a small fraction of heterodimerization-induced fluorescent signals also was detected around the DAPI-stained nuclei. Further studies of subcellular localization of CaHIR1 and its interacting partner proteins, such as Fha1 and CaLRR1 (Choi et al. 2011) in pepper and RPS2 (Qi et al. 2011) in *Arabidopsis*, will be required to understand how plants specifically recognize pathogen-derived molecules or elicitors to activate downstream signals.

Previous studies showed that mutation of the *FhaB* allele reduces adherence of *Erwinia chrysanthemi* and *X. axonopodis* to plant surfaces, thus suppressing the virulence of the plant pathogens (Gottig et al. 2009; Guilhabert and Kirkpatrick 2005; Rojas et al. 2002). Therefore, we investigated whether Fha1 functions as a virulence factor by using a mutant *X. campestris* pv. *vesicatoria* strain carrying the disrupted allele of *fha1* ($\Delta fha1$) and its complemented strain $\Delta fha1$ (*fha1*). As expected, the basal resistance of tomato plants was significantly higher against $\Delta fha1$ infection than against *X. campestris* pv. *vesicatoria* or $\Delta fha1$ (*fha1*) infection. The expression of various *PR* genes was also significantly lower in $\Delta fha1$ -inoculated tomato leaves than in those inoculated with *X. campestris* pv. *vesicatoria* or $\Delta fha1$ (*fha1*). These findings suggest that *fha1* expression is required for the full virulence of *X. campestris* pv. *vesicatoria* and mutation of *fha1* compromised the virulence of *X. campestris* pv. *vesicatoria* in the host tomato plants. However, in pepper plants, we did not detect a significant difference in the HR or bacterial growth between infection with *X. campestris* pv. *vesicatoria* or $\Delta fha1$, suggesting that the ETI of pepper plants is strong enough to suppress the Fha1-induced susceptibility. More recently, *avrBsT* of *X. campestris* pv. *vesicatoria* has been identified as a key effector protein that positively regulates an incompatible interaction between *X. campestris* pv. *vesicatoria* and pepper plants (Kim et al. 2010). Therefore, we used the $\Delta avrBsT$ mutant strain to investigate whether *fha1* functions as a virulence factor in pepper during infection by *X. campestris* pv. *vesicatoria*. As expected, $\Delta avrBsT/\Delta fha1$ showed significantly reduced virulence in terms of reduced bacterial growth and disease severity in pepper leaves. Silencing of *CaHIR1* conferred enhanced resistance of pepper plants to $\Delta avrBsT$ infection. Consistent with our previous studies (Choi et al. 2011), these findings suggest that *CaHIR1* expression contributes to disease susceptibility in plants. More importantly, $\Delta avrBsT$ did not show enhanced virulence in *CaHIR1*-silenced plants compared with $\Delta avrBsT/\Delta fha1$, suggesting the requirement of CaHIR1 for Fha1 to activate *X. campestris* pv. *vesicatoria* virulence. Taken together, these results suggest that the interaction of Fha1 with CaHIR1

plays a pivotal role in *X. campestris* pv. *vesicatoria* virulence during the infection of host plants.

In vitro and in planta attachment assays revealed that the adherence of $\Delta fha1$ to both plastic and plant leaf surfaces was significantly higher than that of the *X. campestris* pv. *vesicatoria* and $\Delta fha1$ (*fha1*) strains. On the other hand, the swarming motility of $\Delta fha1$ was significantly reduced in the SB agar plates. Furthermore, tomato leaves infected with $\Delta fha1$ showed localized necrotic lesions but failed to develop spreading chlorotic symptoms on the entire leaf surface. This suggests that *fha1* expression negatively regulates bacterial adherence but positively regulates *X. campestris* pv. *vesicatoria* motility. Infiltration of the recombinant Fha1 protein into pepper leaves induced callose deposition and cell death but significantly lowered *PR* gene expression compared with the mock-inoculated leaves. These findings suggest that the interaction of CaHIR1 with Fha1 not only induces susceptibility cell death but also actively suppresses *PR* gene expression. Notably, dosage-dependent lowered *PR* gene expression in pepper leaves infiltrated with recombinant Fha1 compared with mock-infiltrated leaves suggests its suppressive role in plant immunity but excludes the phytotoxic effect of Fha1. This was supported by previous results showing that CaHIR1 positively regulates HR and disease-associated cell death but negatively regulates plant defense-related hormone salicylic acid accumulation and *PR* gene expression (Choi et al. 2011). Taking all available evidence together, we suggest that Fha1 enhances pathogen virulence by regulating *X. campestris* pv. *vesicatoria* motility, and suppresses plant basal defense by compromising *PR* gene expression in association with CaHIR1 (Supplementary Fig. S7). Accumulation of Fha1 enhances bacterial motility, increasing the spread of bacteria in plant tissues. Plants also activate disease-associated cell death upon recognition of Fha1 by CaHIR1. Furthermore, Fha1-triggered cell death suppresses *PR* gene expression to overcome the basal defense response in host plants.

MATERIALS AND METHODS

Y2H screening.

The MATCHMAKER GAL4 two-hybrid system (Clontech, Palo Alto, CA, U.S.A.) was used in this study. The genomic DNA library of *X. campestris* pv. *vesicatoria* Bv5-4a was constructed in the GAL4 AD vector (pGADT7). The bait construct was also prepared by cloning the coding region for *CaHIR1* into the GAL4 DNA-binding domain vector (pGBKT7). The *X. campestris* pv. *vesicatoria* prey library and the bait construct were co-transformed into yeast strain AH109 and the transformed yeasts were plated on minimal synthetic dropout media lacking histidine, leucine, and tryptophan. Positive samples were subsequently placed four times on media lacking adenine, histidine, leucine, and tryptophan with X-GAL to monitor the activation of the HIS3, ADE2, and MEL1 reporter genes. Reciprocal switch experiments using the isolated clones were performed to confirm their specific interaction.

Overexpression and purification of Fha1.

The ORF region of *fha1* was cloned into the pET22ba vector (Novagen, San Diego, CA, U.S.A.). Constructs for protein expression were transformed into *Escherichia coli* BL21 (Invitrogen, Carlsbad, CA, U.S.A.) and grown overnight in 5 ml of Luria-Bertani (LB) medium at 37°C. An aliquot of the culture was added to 50 ml of LB broth and grown at 37°C to an optical density at 600 nm (OD_{600}) = 0.5. Protein expression was induced with 1 mM isopropyl- β -D-thio-galactoside for 12 h at 28°C. Bacterial cells harvested by centrifugation were resuspended in 1× PBS buffer (pH 8.0) and then disrupted by soni-

cation. The lysate was centrifuged for 1 h at $15,000 \times g$ and the supernatant was collected. To purify His-tagged FhaI protein, the soluble fraction was loaded onto a Ni-NTA column (GE Healthcare, Piscataway, NJ, U.S.A.) pre-equilibrated with $1 \times$ PBS buffer. The FhaI was eluted with $1 \times$ PBS buffer containing 500 mM imidazole. The concentration of purified FhaI was determined by the Bradford assay (Bradford 1976).

Coimmunoprecipitation.

To overexpress *CaHIR1* (Choi et al. 2011; Jung and Hwang 2007) in pepper plants, cMyc-tagged *CaHIR1* was cloned into the binary vector pBIN35S and transformed into the *Agrobacterium tumefaciens* GV3101. *A. tumefaciens* GV3101 containing empty vector or the pBIN35S:*CaHIR1* construct was grown overnight at 28°C in yeast-peptone media (yeast extract at 10 g/liter, peptone at 10 g/liter, and NaCl [Difco, Detroit] at 5 g/liter, pH 7.5, adjusted with NaOH) containing kanamycin (Kan) at $50 \mu\text{g ml}^{-1}$ and rifampicin (Rif) at $100 \mu\text{g ml}^{-1}$. The bacterial cells collected by centrifugation were suspended in infiltration buffer (10 mM MgCl_2 , 10 mM 2-[N-morpholino] ethane sulfonic acid [pH 5.7], and 200 μM acetosyringone) to a final OD_{600} of 0.5 and incubated for 1 h at room temperature. Bacterial suspensions were infiltrated into the abaxial side of pepper leaves with a needleless syringe. Total protein extracts were prepared by grinding 1 g of pepper leaf tissue in 2 ml of lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 10 mM EDTA, 0.2% Triton X-100, and complete protease inhibitor cocktail [Roche, Mannheim, Germany]). Insoluble materials were pelleted by centrifugation at $20,000 \times g$ for 20 min at 4°C. The supernatant (500 μl) was combined with 100 μl of resuspended anti-cMyc affinity matrix (Roche) with or without 1 μg of His-tagged FhaI protein. After incubation at 4°C overnight, the resins were washed four times with 1 ml of lysis buffer and subjected to centrifugation at $15,000 \times g$ at 4°C. The washed pellets were added with 200 μl of electrophoresis sample buffer (20 mM Tris-HCl [pH 7.5], 2 mM EDTA, 5% sodium dodecyl sulfate [SDS], 0.02% bromophenol blue, 20% glycerol, and 200 mM dithiothreitol), boiled for 5 min, and then centrifuged at $15,000 \times g$ at 4°C. Supernatant (20 μl) was subjected to SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane for immunoblotting using anti-His antibody (Sigma-Aldrich, St. Louis).

BiFC analyses.

BiFC experiments were conducted as previously described (Choi et al. 2012; Walter et al. 2004). Briefly, the coding regions of *fhaI* and *CaHIR1* were cloned into pSPYNE-35S and pSPYCE-35S. *A. tumefaciens* GV3101 carrying the BiFC constructs was coinfiltrated into the abaxial side of *N. benthamiana* leaves ($\text{OD}_{600} = 0.5$ for each construct). The epidermal cell layers were observed by confocal laser-scanning microscopy (LSM 5 Exciter) 1 and 2 days after agroinfiltration.

Bacterial strains and growth conditions.

E. coli DH5 α was cultured overnight in LB medium at 37°C. *X. campestris* pv. *vesicatoria* Bv5-4a and mutant $\Delta fhaI$ and $\Delta fhaI$ (*fhaI*) strains were cultured overnight in YN medium (yeast extract at 5 g liter $^{-1}$ and nutrient broth at 8 g liter $^{-1}$) at 28°C. *P. syringae* pv. *tomato* DC3000 was grown overnight at 28°C on King's B medium (protease peptone at 10 g liter $^{-1}$, K_2HPO_4 at 1.5 g liter $^{-1}$, glycerol at 15 g liter $^{-1}$, and 5 mM MgSO_4 , pH 7.0). Antibiotics used were Rif at $50 \mu\text{g ml}^{-1}$, Kan at $50 \mu\text{g ml}^{-1}$, and spectinomycin (Sp) at $50 \mu\text{g ml}^{-1}$.

Site-directed mutagenesis and complementation.

The 379-bp N-terminal fragment of *fhaI* was amplified by PCR. The amplified fragment of *fhaI* was digested with *EcoRI*

and *XhoI* and cloned into the pKnockout-G vector. The resulting plasmid was transformed into *E. coli* and used for triparental mating. The donor *E. coli* strain carrying pKnockout-G:*fhaI*, helper *E. coli* strain carrying pRK2013, and recipient *X. campestris* pv. *vesicatoria* Bv5-4a were combined in a cell mixture and spread over YN agar plates without antibiotics (Windgassen et al. 2000). After overnight incubation at 28°C, the exconjugants were washed from the plates and selected on YN agar medium supplemented with Rif and Sp at $50 \mu\text{g ml}^{-1}$ each. To complement the $\Delta fhaI$ mutant, the genomic regions containing the C-terminal regions of the gene encoding a hypothetical protein and full-length *fhaI* were amplified by PCR. The amplified fragment was digested with *EcoRI* and cloned into the pVIK112 vector (Kalogeraki and Winans 1997). The resulting plasmid was transformed into *E. coli* and used for triparental mating, which was performed as described above using the $\Delta fhaI$ as a recipient strain.

RT-PCR and quantitative real-time RT-PCR analyses.

To examine the expression of *fhaI* or plant *PR* genes, total RNA was isolated from *X. campestris* pv. *vesicatoria* cells or plant tissues, respectively, using the TRIzol reagent according to the manufacturer's instructions (Invitrogen). After DNase treatment, RT reactions were performed with total RNA (2 μg) and oligo p(dN) $_6$ primer at 42°C using AMV reverse transcriptase in a 20- μl reaction volume (Roche). To analyze the expression of *fhaI*, aliquots (1 μl) of RT reaction products were used for RT-PCR analysis. RT-PCR conditions were 95°C for 10 min and 30 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 1 min. PCR products were analyzed by agarose gel electrophoresis to confirm the presence of single bands. The expression of plant *PR* genes was examined by quantitative real-time RT-PCR. First-strand cDNA was diluted 1:10 with distilled water and used for real-time RT-PCR with iQ SYBR Green Supermix (Bio-Rad, Munich) using iCycler iQ (Bio-Rad). Tomato *ACTIN* and pepper *18S rRNA* were used as the internal controls to normalize *PR* gene transcript levels.

Adhesion assays.

To examine the adhesion of cells to a plastic surface, overnight cultures of the *X. campestris* pv. *vesicatoria*, $\Delta fhaI$, and $\Delta fhaI$ (*fhaI*) strains in YN media were collected by centrifugation and resuspended in phosphate buffer (pH 7.0) to an OD_{600} of 0.6. Aliquots of 200 μl of each bacterial suspension were transferred to each well of a 96-well PVC plate and incubated for 6 h at 28°C. Bacterial adherence was measured by staining with 1% CV for 15 min at room temperature after repetitive washing of the plate to remove nonadherent cells. Excess stain was removed by repetitive washing of the plate under running tap water. The CV stain was solubilized by the addition of 150 μl of 95% ethanol to each well and staining was quantified with a microplate reader at 570-nm absorption wavelength. To measure the adherence of bacteria to the leaf surface of tomato and pepper plants, 20 μl of each bacterial suspension collected from the abaxial side of leaves was incubated for 6 h at 28°C in a humidified chamber. Bacterial adhesion was measured by CV staining as described above.

Plant materials and pathogen inoculation.

Seed of pepper (*C. annuum* L. 'Nockwang') and tomato (*Solanum lycopersicon* L. 'Kwang-bok') were germinated and grown in pots containing steam-sterilized soil mix (peat moss, perlite, and vermiculite; 5:3:2, vol/vol/vol) and loam soil (1:1, vol/vol) at 25°C with a photoperiod of 16 h of light and 8 h of darkness at a light intensity of $100 \mu\text{mol photons m}^{-2} \text{s}^{-1}$.

WT and mutant *X. campestris* pv. *vesicatoria* strains were cultured overnight in YN broth at 28°C. Prior to inoculation,

bacterial cells were collected by centrifugation and resuspended in 10 mM MgCl₂ solution (10⁸ CFU ml⁻¹). Bacterial suspensions were infiltrated into fully expanded leaves using a needleless syringe. The infected plants were incubated at 28°C and harvested at various time points for bacterial growth assay.

Generation of antibodies against Fha1.

Rabbit polyclonal antibodies against the Fha1-specific peptide SVLNDSETNRYNSR were generated by AbFrontier (Seoul, Korea) and used to detect Fha1 proteins.

Histochemical staining.

Trypan blue and aniline blue staining were performed as previously described (Choi et al. 2011). To visualize the cell death response, leaves were stained with trypan blue and destained with a saturated chloral hydrate solution (2.5 g ml⁻¹). For aniline blue staining, leaves were cleared in alcoholic lactophenol (1 ml of lactic acid, 1 ml of glycerol, 1 g of phenol, and 1 ml of H₂O). Cleared leaves were stained with 0.01% aniline blue in 0.15 M phosphate buffer, pH 9.5. Callose deposition was visualized with a fluorescence microscope under UV light (BH-2; Olympus, Tokyo).

For visualization of the nuclei, detached leaves were immersed in a staining solution (0.1% DAPI in 5% dimethyl sulfoxide [DMSO]). DAPI fluorescence images were obtained using excitation at 405 nm and emission at 435 to 480 nm.

As a reliable tracer for endocytic trafficking, the lipophilic styryl dye FM4-64 (Invitrogen) was resuspended in DMSO to a concentration of 5 mM, diluted at 1:500 to 1:1000 in water, and infiltrated into the abaxial side of *N. benthamiana* leaves.

Virus-induced gene silencing.

The TRV-based virus-induced gene silencing system was used for gene silencing in pepper as described previously (Choi et al. 2013; Kim and Hwang 2011; Liu et al. 2002). The 225-bp 5' region of *CaHIR1* was amplified by PCR using specific primers and inserted into pTRV2 to generate TRV2: *CaHIR1*. *A. tumefaciens* GV3101 strains carrying pTRV1 or pTRV2 constructs were coinfiltrated into the fully expanded cotyledons of pepper (final OD₆₀₀ = 0.2 for each construct). Plants were placed in a growth room at 25°C with a photoperiod cycle of 16 h of light and 8 h of darkness to allow growth and viral spread. Plants silenced for 4 to 5 weeks were used for pathogen inoculation.

Arabidopsis transformation.

The *fha1* coding region was PCR-amplified and cloned into the *Xba*I and *Bam*HI sites of the binary vector pBIN35S (Choi and Hwang 2011). The resulting binary plasmid was transformed into *Agrobacterium* GV3101 by electroporation. The *Agrobacterium*-mediated transformation was performed using the floral-dipping method (Clough and Bent 1998). Transgenic plants were selected by germinating seed on Murashige and Skoog agar medium containing Kan at 50 µg ml⁻¹.

LITERATURE CITED

Altenbach, D., and Robatzek, S. 2007. Pattern recognition receptors: From the cell surface to intracellular dynamics. *Mol. Plant-Microbe Interact.* 20:1031-1039.

Bent, A. F., and Mackey, D. 2007. Elicitors, effectors, and *R* genes: The new paradigm and a lifetime supply of questions. *Annu. Rev. Phytopathol.* 45:399-436.

Bolte, S., Talbot, C., Boutte, Y., Catrice, O., Read, N.D., and Satiat-Jeunemaitre, B. 2004. FM-dyes as experimental probes for dissecting vesicle trafficking in living plant cells. *J. Microsc.* 214:159-173.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-254.

Browman, D. T., Hoegg, M. B., and Robbins, S. M. 2007. The SPFH domain-containing proteins: More than lipid raft markers. *Trends Cell Biol.* 17:394-402.

Chinchilla, D., Bauer, Z., Regenass, M., Boller, T., and Felix, G. 2006. The *Arabidopsis* receptor kinase FLS2 binds flg22 and determines the specificity of flagellin perception. *Plant Cell* 18:465-476.

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.

Choi, D. S., and Hwang, B. K. 2011. Proteomics and functional analyses of pepper *abscisic acid-responsive1* (*ABR1*), which is involved in cell death and defense signaling. *Plant Cell* 23:823-842.

Choi, D. S., Hwang, I. S., and Hwang, B. K. 2012. Requirement of the cytosolic interaction between pathogenesis-related protein10 and leucine-rich repeat protein1 for cell death and defense signaling in pepper. *Plant Cell* 24:1675-1690.

Choi, H. W., Kim, Y. J., Lee, S. C., Hong, J. K., and Hwang, B. K. 2007. Hydrogen peroxide generation by the pepper extracellular peroxidase CaPO2 activates local and systemic cell death and defense response to bacterial pathogens. *Plant Physiol.* 145:890-904.

Choi, H. W., Kim, Y. J., and Hwang, B. K. 2011. The hypersensitive induced reaction and leucine-rich repeat proteins regulate plant cell death associated with disease and plant immunity. *Mol. Plant-Microbe Interact.* 24:68-78.

Choi, H. W., Kim, N. H., Lee, Y. K., and Hwang, B. K. 2013. The pepper extracellular xyloglucan-specific endo-β-1,4-glucanase inhibitor protein gene, *CaXEGIP1*, is required for plant cell death and defense responses. *Plant Physiol.* 161:384-396.

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

Cunnac, S., Wilson, A., Nuwer, J., Kirik, A., Baranage, G., and Mudgett, M. B. 2007. A conserved carboxylesterase is a SUPPRESSOR OF AVRBT-ELICITED RESISTANCE in *Arabidopsis*. *Plant Cell* 19:688-705.

Felix, G., and Boller, T. 2003. Molecular sensing of bacteria in plants. The highly conserved RNA-binding motif RNP-1 of bacterial cold shock proteins is recognized as an elicitor signal in tobacco. *J. Biol. Chem.* 278:6201-6208.

Flor, H. H. 1971. Current status of the gene-for-gene concept. *Annu. Rev. Phytopathol.* 9:275-296.

Gadea, J., Mayda, M. E., Conejero, V., and Vera, P. 1996. Characterization of defense-related genes ectopically expressed in viroid-infected tomato plants. *Mol. Plant-Microbe Interact.* 9:409-415.

Gao, M., Wang, X., Wang, D., Xu, F., Ding, X., Zhang, Z., Bi, D., Cheng, Y. T., Chen, S., Li, X., and Zhang, Y. 2009. Regulation of cell death and innate immunity by two receptor-like kinases in *Arabidopsis*. *Cell Host Microbe* 6:34-44.

Goebel, E. M., Zhang, X., and Harvill, E. T. 2009. *Bordetella pertussis* infection or vaccination substantially protects mice against *B. bronchiseptica* infection. *PLoS One* 4:e6778.

Göhre, V., Spallek, T., Häweker, H., Mersmann, S., Mentzel, T., Boller, T., de Torres, M., Mansfield, J. W., and Robatzek, S. 2008. Plant pattern-recognition receptor FLS2 is directed for degradation by the bacterial ubiquitin ligase AvrPtoB. *Curr. Biol.* 18:1824-1832.

Gómez-Gómez, L., Bauer, Z., and Boller, T. 2001. Both the extracellular leucine-rich repeat domain and the kinase activity of FLS2 are required for flagellin binding and signaling in *Arabidopsis*. *Plant Cell* 13:1155-1163.

Gordon, S. 2002. Pattern recognition receptors: Doubling up for the innate immune response. *Cell* 111:927-930.

Gottig, N., Garavaglia, B. S., Garofalo, C. G., Orellano, E. G., and Ottado, J. 2009. A filamentous hemagglutinin-like protein of *Xanthomonas axonopodis* pv. *citri*, the phytopathogen responsible for citrus canker, is involved in bacterial virulence. *PLoS One* 4:e4358.

Guilhabert, M. R., and Kirkpatrick, B. C. 2005. Identification of *Xylella fastidiosa* antivirulence genes: Hemagglutinin adhesins contribute a biofilm maturation to *X. fastidiosa* and colonization and attenuate virulence. *Mol. Plant-Microbe Interact.* 18:856-868.

Ishibashi, Y., Claus, S., and Relman, D. A. 1994. *Bordetella pertussis* filamentous hemagglutinin interacts with a leukocyte signal transduction complex and stimulates bacterial adherence to monocyte CR3 (CD11b/CD18). *J. Exp. Med.* 180:1225-1233.

Jones, J. D. G., and Dangl, J. L. 2006. The plant immune system. *Nature* 444:323-329.

Jung, E. H., Jung, H. W., Lee, S. C., Han, S. W., Heu, S., and Hwang, B. K. 2004. Identification of a novel pathogen-induced gene encoding a leucine-rich repeat protein expressed in phloem cells of *Capsicum annuum*. *Biochim. Biophys. Acta.* 1676:211-222.

Jung, H. W., and Hwang, B. K. 2007. The leucine-rich repeat (LRR) protein, CaLRR1, interacts with the hypersensitive induced reaction (HIR)

- protein, CaHIR1, and suppresses cell death induced by CaHIR1 protein. *Mol. Plant Pathol.* 8:503-514.
- Jung, H. W., Lim, C. W., Lee, S. C., Choi, H. W., Hwang, C. H., and Hwang, B. K. 2008. Distinct roles of the pepper hypersensitive induced reaction protein gene *CaHIR1* in disease and osmotic stress, as determined by comparative transcriptome and proteome analyses. *Planta* 227:409-425.
- Kajava, A. V., Cheng, N., Cleaver, R., Kessel, M., Simon, M. N., Willery, E., Jacob-Dubuisson, F., Loch, C., and Steven, A. C. 2001. Beta-helix model for the filamentous haemagglutinin adhesin of *Bordetella pertussis* and related bacterial secretory proteins. *Mol. Microbiol.* 42:279-292.
- Kalogeraki, V. S., and Winans, S. C. 1997. Suicide plasmids containing promoterless reporter genes can simultaneously disrupt and create fusions to target genes of diverse bacteria. *Gene* 188:69-75.
- Kang, H. G., Oh, C. S., Sato, M., Katagiri, F., Glazebrook, J., Takahashi, H., Kachroo, P., Martin, G. B., and Klessig, D. F. 2010. Endosome-associated CRT1 functions early in resistance gene-mediated defense signaling in *Arabidopsis* and tobacco. *Plant Cell* 22:918-936.
- Kankanala, P., Czymmek, K., and Valent, B. 2007. Roles for rice membrane dynamics and plasmodesmata during biotrophic invasion by the blast fungus. *Plant Cell* 19:706-724.
- Kim, D. S., and Hwang, B. K. 2011. The pepper receptor-like cytoplasmic protein kinase CaPIK1 is involved in plant signaling of defense and cell-death responses. *Plant J.* 66:642-655.
- Kim, N. H., Choi, H. W., and Hwang, B. K. 2010. *Xanthomonas campestris* pv. *vesicatoria* effector AvrBsT induces cell death in pepper, but suppresses defense responses in tomato. *Mol. Plant-Microbe Interact.* 23:1069-1082.
- Klarzynski, O., Plesse, B., Joubert, J. M., Yvin, J. C., Kopp, M., Kloareg, B., and Fritig, B. 2000. Linear beta-1,3 glucans are elicitors of defense responses in tobacco. *Plant Physiol.* 124:1027-1038.
- Kunze, G., Zipfel, C., Robatzek, S., Niehaus, K., Boller, T., and Felix, G. 2004. The N terminus of bacterial elongation factor Tu elicits innate immunity in *Arabidopsis* plants. *Plant Cell* 16:3496-3507.
- Link, S., Schmitt, K., Beier, D., and Gross, R. 2007. Identification and regulation of expression of a gene encoding a filamentous hemagglutinin-related protein in *Bordetella holmesii*. *BMC Microbiol.* 7:100.
- Liu, Y., Schiff, M., and Dinesh-Kumar, S. P. 2002. Virus-induced gene silencing in tomato. *Plant J.* 31:777-786.
- Locht, C., Antoine, R., and Jacob-Dubuisson, F. 2001. *Bordetella pertussis*, molecular pathogenesis under multiple aspects. *Curr. Opin. Microbiol.* 4:82-89.
- Mobberley-Schuman, P. S., and Weiss, A. A. 2005. Influence of CR3 (CD11b/CD18) expression on phagocytosis of *Bordetella pertussis* by human neutrophils. *Infect. Immun.* 73:7317-7323.
- Nürnberg, T., Brunner, F., Kemmerling, B., and Piater, L. 2004. Innate immunity in plants and animals: Striking similarities and obvious differences. *Immunol. Rev.* 198:249-266.
- Qi, Y., and Katagiri, F. 2012. Membrane microdomain may be a platform for immune signaling. *Plant Signal Behav.* 7:454-456.
- Qi, Y., Tsuda, K., Nguyen, V., Wang, X., Lin, J., Murphy, A. S., Glazebrook, J., Thordal-Christensen, H., and Katagiri, F. 2011. Physical association of *Arabidopsis* hypersensitive induced reaction proteins (HIRs) with the immune receptor RPS2. *J. Biol. Chem.* 286:31297-3307.
- Relman, D., Tuomanen, E., Falkow, S., Golenbock, D. T., Saukkonen, K., and Wright, S. D. 1990. Recognition of a bacterial adhesion by an integrin: Macrophage CR3 ($\alpha_M\beta_2$, CD11b/CD18) binds filamentous hemagglutinin of *Bordetella pertussis*. *Cell* 61:1375-1382.
- Rieber, N., Graf, A., Belohradsky, B. H., Hartl, D., Urschel, S., Riffelmann, M., Wirsing von König, C. H., and Liese, J. 2008. Differences of humoral and cellular immune response to an acellular pertussis booster in adolescents with a whole cell or acellular primary vaccination. *Vaccine* 26:6929-6935.
- Robatzek, S., Chinchilla, D., and Boller, T. 2006. Ligand-induced endocytosis of the pattern recognition receptor FLS2 in *Arabidopsis*. *Genes Dev.* 20:537-542.
- Rojas, C. M., Ham, J. H., Deng, W. L., Doyle, J. J., and Collmer, A. 2002. HecA, a member of a class of adhesins produced by diverse pathogenic bacteria, contributes to the attachment, aggregation, epidermal cell killing, and virulence phenotypes of *Erwinia chrysanthemi* EC16 on *Nicotiana glauca* seedlings. *Proc. Natl. Acad. Sci. U.S.A.* 99:13142-13147.
- van den Heuvel, K. J., Hulzink, J. M., Barendse, G. W., and Wullems, G. J. 2001. The expression of tgas118, encoding a defensin in *Lycopersicon esculentum*, is regulated by gibberellin. *J. Exp. Bot.* 52:1427-1436.
- Walter, M., Chaban, C., Schütze, K., Batistic, O., Weckermann, K., Näke, C., Blazevic, D., Grefen, C., Schumacher, K., Oecking, C., Harter, K., and Kudla, J. 2004. Visualization of protein interactions in living plant cells using bimolecular fluorescence complementation. *Plant J.* 40:428-438.
- Weingart, C. L., and Weiss, A. A. 2000. *Bordetella pertussis* virulence factors affect phagocytosis by human neutrophils. *Infect. Immun.* 68:1735-1739.
- Windgassen, M., Urban, A., and Jaeger, K. E. 2000. Rapid gene inactivation in *Pseudomonas aeruginosa*. *FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Lett.* 193:201-215.
- Zipfel, C. 2008. Pattern-recognition receptors in plant innate immunity. *Curr. Opin. Immunol.* 20:10-16.
- Zipfel, C., and Felix, G. 2005. Plants and animals: A different taste for microbes? *Curr. Opin. Plant Biol.* 8:353-360.
- Zipfel, C., Kunze, G., Chinchilla, D., Caniard, A., Jones, J. D., Boller, T., and Felix, G. 2006. Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts *Agrobacterium*-mediated transformation. *Cell* 125:749-760.

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