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Article

Pseudomonas syringae Type III Effector HopZ1 Targets a Host Enzyme to Suppress Isoflavone Biosynthesis and Promote Infection in Soybean

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SUMMARY

Type III secreted effectors (T3SEs), such as *Pseudomonas syringae* HopZ1, are essential bacterial virulence proteins injected into the host cytosol to facilitate infection. However, few direct targets of T3SEs are known. Investigating the target(s) of HopZ1 in soybean, a natural *P. syringae* host, we find that HopZ1 physically interacts with the isoflavone biosynthesis enzyme, 2-hydroxyisoflavanone dehydratase (GmHID1). *P. syringae* infection induces *gmhid1* expression and production of daidzein, a major soybean isoflavone. Silencing *gmhid1* increases susceptibility to *P. syringae* infection, supporting a role for GmHID1 in innate immunity. *P. syringae* expressing active but not the catalytic mutant of HopZ1 inhibits daidzein induction and promotes bacterial multiplication in soybean. HopZ1-enhanced *P. syringae* multiplication is at least partially dependent on GmHID1. Thus, GmHID1 is a virulence target of HopZ1 to promote *P. syringae* infection of soybean. This work highlights the isoflavonoid biosynthesis pathway as an antibacterial defense mechanism and a direct T3SE target.

INTRODUCTION

Gram-negative bacterial pathogens rely on the type III secretion system (T3SS) and the type III secreted effectors (T3SEs) to cause diseases in animals and plants. T3SEs are essential virulence proteins delivered directly from bacteria into the host cytoplasm. Once inside the host cells, T3SEs target specific host substrates to promote bacterial colonization and multiplication. In plant pathogens, one of the most important functions of T3SEs is to suppress host immunity (Grant et al., 2006; Guo et al., 2009).

The immune responses protecting plants from potential pathogens are generally initiated with the recognition of pathogen-associated or microbe-associated molecular patterns (PAMPs or MAMPs) (Chisholm et al., 2006; Jones and Dangl, 2006). The perception of these conserved molecular patterns triggers PAMP-triggered immunity (PTI), which effectively restricts the growth of the vast majority of potential pathogens in plants (Boller and He, 2009; Jones and Dangl, 2006). A second layer of plant defense is resistance (R) protein-mediated immunity. R proteins are evolved to detect specific T3SEs, or their functions, and trigger a localized and robust programmed cell death referred to as the hypersensitive response (HR) (Greenberg, 1997; Heath, 2000). As a result, pathogen multiplication and spread in plants is restricted by this effector-triggered immunity (ETI) (Dangl and Jones, 2001).

Many T3SEs are able to suppress PTI and/or ETI in plant cells (Grant et al., 2006; Guo et al., 2009), although how they accomplish this function remains largely unknown. A critical step to answer this question is the identification and characterization of the host targets of individual T3SEs (Alfano, 2009). In the plant pathogen *Pseudomonas syringae*, the virulence targets of a few T3SEs, including AvrPto, AvrPtoB, AvrB, AvrPphB, HopM1, HopU1, HopAI1, and HopI1, have been characterized. These effectors suppress immune responses by interfering with a variety of biological processes in plant hosts. For example, AvrPto, AvrPtoB, AvrB, AvrPphB, and HopAI1 directly block or interfere with defense signal transduction (Cui et al., 2010; Gimenez-Ibanez et al., 2009; Gohre et al., 2008; He et al., 2006; Rosebrock et al., 2007; Shan et al., 2008; Xiang et al., 2008; Zhang et al., 2007, 2010); HopM1 interferes with vesicle trafficking (Nomura et al., 2006); HopU1 alters RNA metabolism (Fu et al., 2007); and HopI1 targets a plant heat shock protein in chloroplasts to promote pathogenicity (Jelenska et al., 2010). To date, the precise roles of the majority of the T3SEs in pathogenesis are unknown.

One of the most widely distributed and diverse T3SE families in bacteria is the YopJ family of cysteine proteases/acetyltransferases (Ma et al., 2006). YopJ family T3SEs all share a conserved catalytic core, consisting of three key amino acid residues (histidine, glutamic acid, and cysteine), that is identical to the clan CE (C55 family) cysteine proteases (Barrett and Rawlings, 2001). However, YopJ has been shown to possess an acetyltransferase activity in animals and block the activation of

mitogen-activated protein kinases through acetylation of the serine and threonine residues in their activation loops (Mukherjee et al., 2006; Orth, 2002). So far, more than ten YopJ homologs have been identified in plant pathogens including *Xanthomonas*, *Ralstonia*, *Erwinia*, and *Pseudomonas* (Ma et al., 2006). Experimental evidence suggests that they may have different enzymatic activities. For example, AvrXv4 in *Xanthomonas campestris* pv. vesicatoria has desumolyation activity (Roden et al., 2004). HopZ1, HopZ2, HopZ3 in *P. syringae*, and AvrBsT in *X. campestris* have weak in vitro cysteine protease activity (Ma et al., 2006; Szczesny et al., 2010). Recently, PopP2 in *Ralstonia solanacearum* was reported to have autoacetylation activity on a lysine residue (Tasset et al., 2010). No matter which enzymatic activity these YopJ-like T3SEs possess, the intact catalytic triads are required for their cellular functions.

HopZ1 of *P. syringae* is a YopJ-like T3SE with two full-length alleles (HopZ1a and HopZ1b), one truncated allele (HopZ1c), and several degenerated alleles identified from a variety of *P. syringae* isolates (Ma et al., 2006). Remarkably, all the *P. syringae* pv. glycinea (*Pgy*) strains that have tested produce a functional HopZ1b allele (Ma et al., 2006). Since *Pgy* strains are the causal agents of bacterial blight disease on soybean (*Glycine max* [L.] Merr.), we have been using the natural soybean—*Pgy* pathosystem to investigate the cellular function of HopZ1. We previously demonstrated that HopZ1b$_{PgyBR1}$ (hereafter HopZ1b) promotes *P. syringae* multiplication in soybean, whereas the closely related HopZ1a$_{PsyA2}$ (hereafter HopZ1a) triggers HR (Zhou et al., 2009). Mutants with the catalytic cysteine residues (C216 in HopZ1a or C212 in HopZ1b) replaced with alanines are abrogated in the virulence function of HopZ1b and the cell death-triggering activity of HopZ1a, indicating that the cellular functions of HopZ1 are accomplished through their enzymatic activity (Ma et al., 2006; Zhou et al., 2009).

The goal of this study was to gain insights into the molecular basis of the function of HopZ1 by the characterization of HopZ1 substrates in soybean. Since HopZ1 potentially possesses a cysteine protease/acetyltransferase activity, we hypothesize that HopZ1 modifies specific protein(s) in plant hosts to promote bacterial infection. To test this hypothesis, the host target(s) of HopZ1a and HopZ1b was identified in soybean using yeast two-hybrid screens. We find that HopZ1 physically interacts with an isoflavone biosynthesis enzyme, 2-hydroxyisoflavanone dehydratase (GmHID1), and provide evidence that GmHID1 is a major virulence target of HopZ1 in order to facilitate *P. syringae* infection in soybean.

RESULTS

Identification of HopZ1-Interacting Proteins in Soybean

HopZ1-associating proteins were identified by using yeast two-hybrid screens (Matchmaker, Clontech) with HopZ1a and HopZ1b as the baits and a soybean cDNA library as the prey. Five soybean proteins were found to strongly interact with both HopZ1a and HopZ1b. An additional four proteins interacted with either HopZ1a or HopZ1b. The catalytic mutants HopZ1a (C216A) and HopZ1b(C212A) interacted with all these HopZ1-associating proteins in the same way as their corresponding wild-type proteins (data not shown).

One protein identified from the yeast two-hybrid screens that interacts with both HopZ1 alleles is the enzyme 2-hydroxyisoflavanone dehydratase (GmHID1, gm01 g45020), one of the biosynthetic enzymes for isoflavonoids production in soybean (Akashi et al., 2005) (Figure 1A). In soybean, GmHID1 catalyzes the reaction converting 2-hydroxyisoflavanones to isoflavones, mainly daidzein and genistein (Akashi et al., 2005). Because isoflavones are important secondary metabolites during plant-microbe interactions, we selected GmHID1 for further analysis.

A search in the soybean genome sequence revealed an open reading frame (gm11 g00650) as a homolog of GmHID1—we named it GmHID2. GmHID2 shares 95% similarity with GmHID1 in the amino acid sequences, but it is 33 amino acids shorter at the N terminus. So far, no enzymatic activity has been defined from GmHID2. Yeast two-hybrid assays showed that unlike GmHID1, GmHID2 did not interact with HopZ1 (see Figure S1A available online). GmHID2 was then used as a negative control in experiments characterizing HopZ1-GmHID1 interactions in planta. Furthermore, the N-terminal 64 aa of HopZ1a is sufficient and necessary to mediate the interaction with GmHID1, whereas the full length of GmHID1 is required for its interaction with HopZ1 (Figure S1B).

HopZ1 Interacts with GmHID1 In Vitro and In Vivo

Physical interactions between HopZ1 and GmHID1 were verified using a series of in vivo and in vitro assays. Recombinant HopZ1-GST and GmHID1-FLAG proteins were overexpressed in *E. coli* strain BL21(DE3). Whole lysates of HopZ1a-GST, HopZ1b-GST, or GST were incubated with an equal amount of the whole lysate of GmHID1-FLAG and purified with glutathione GST-binding resin. Both HopZ1a-GST- and HopZ1b-GST-bound resins, but not GST-bound resins, provided enrichment of GmHID1-FLAG (Figure 1B), indicating that HopZ1a and HopZ1b interacted with GmHID1 in vitro.

We then examined the subcellular localization of GmHID1 to determine whether it overlaps with the localization of HopZ1 in plant cells. Colocalization of GmHID1 and HopZ1 would indicate that these proteins could interact in planta. HopZ1 and GmHID1 were fused, at their C termini, to yellow fluorescent protein (YFP), and expressed in *Nicotiana benthamiana* by *Agrobacterium*-mediated transient expression. Protein localizations were indicated by the fluorescence generated by the YFP fusion proteins in the pavement cells of the infiltrated leaves 24 hr post-inoculation (hpi). GmHID1-YFP appeared to be present mainly in the cytoplasm with some yellow fluorescence appearing in the nucleus (Figure 1C). This is consistent with the subcellular localization of several other enzymes functioning in isoflavonoid biosynthesis, such as chalcone synthase (CHS) and chalcone isomerase (CHI) in *Arabidopsis* and (iso)flavonoid malonytransferase in *Medicago truncatula* (Saslowsky et al., 2005; Yu et al., 2008). As reported previously (Lewis et al., 2008; Zhou et al., 2009), HopZ1-YFP was mainly associated with the plasma membrane. We also observed the presence of HopZ1-YFP in the cytoplasm and nucleus (Figure 1C). These data suggest that HopZ1 and GmHID1 could physically interact with each other in plant cells.

To confirm the interaction between HopZ1 and GmHID1 in planta, we performed bimolecular fluorescence complementation (BiFC) assays. HopZ1 and GmHID1 were fused with the

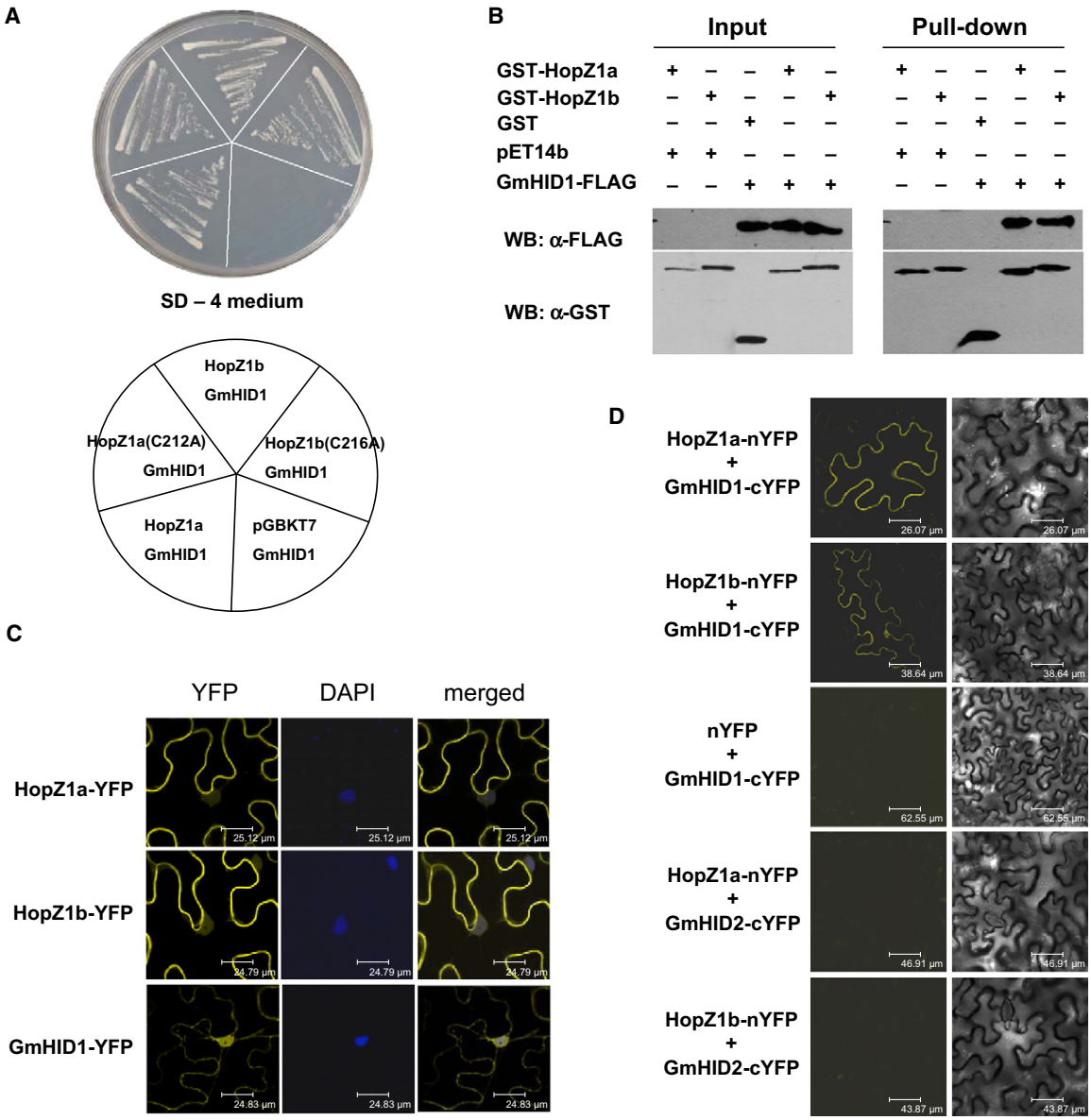

Figure 1. HopZ1 Interacts with Soybean Protein GmHID1

(A) HopZ1 interacts with GmHID1 in yeast. Yeast strain AH109 was transformed with the prey plasmid pGADT7::GmHID1 and one of the following bait plasmids: empty vector pGBKT7, pGBKT7::HopZ1a, pGBKT7::HopZ1a(C216A), pGBKT7::HopZ1b, and pGBKT7::HopZ1b(C212A). Yeast transformants were grown on the selective minimal SD medium lacking adenine, tryptophan, histidine, and leucine (−4). The plates were photographed 2 days after inoculation. (See also Figure S1.)

(B) In vitro pull-down analysis of HopZ1 and GmHID1. HopZ1-GST- or GST-bound glutathione GST-binding resins were incubated with equal amount of cell lysate containing GmHID1-FLAG. The precipitation of GmHID1-FLAG with the GST-binding resins was examined by western blots using anti-FLAG antibody before (Input) and after washes (Pull-down). The same protein gel was blotted with anti-GST antibody to show that equal amount of GST- or GST-HopZ1 was present in the protein mixtures.

(C) Subcellular localization of GmHID1 and HopZ1 in planta. In planta localization of HopZ1a, HopZ1b, and GmHID1 were examined in *Nicotiana benthamiana* leaves inoculated with *Agrobacterium tumefaciens* strain C58C1(pCH32) delivering GmHID1-YFP, HopZ1a-YFP, or HopZ1b-YFP. Fluorescences (shown in the left column) were examined using confocal microscopy (60×) 24 hr postinfiltration. The cells were stained with diamidin-2-phenylindol (DAPI) for nuclei (the middle column), and the merged images are shown in the right column.

(D) BiFC analysis of HopZ1/GmHID1 interactions in *N. benthamiana*. The catalytic mutants HopZ1(C/A) were used in this analysis because the wild-type HopZ1a and HopZ1b triggered HR in *N. benthamiana*. HopZ1a(C/A) fused with the nonfluorescent nYFP and GmHID1 fused with cYFP were coexpressed in *N. benthamiana* through *Agrobacterium*-mediated transient expression. GmHID2 fused with cYFP was used as a negative control. Forty-eight hours postinoculation, fluorescence was examined by confocal microscopy from the infected leaves coexpressing one of the following combinations of proteins: GmHID1-cYFP+HopZ1a(C216A)-nYFP, GmHID1-cYFP+HopZ1b(C212A)-nYFP, GmHID1-cYFP+ nYFP, GmHID2-cYFP+HopZ1a(C216A)-nYFP, and GmHID2-cYFP+HopZ1b(C212A)-nYFP.

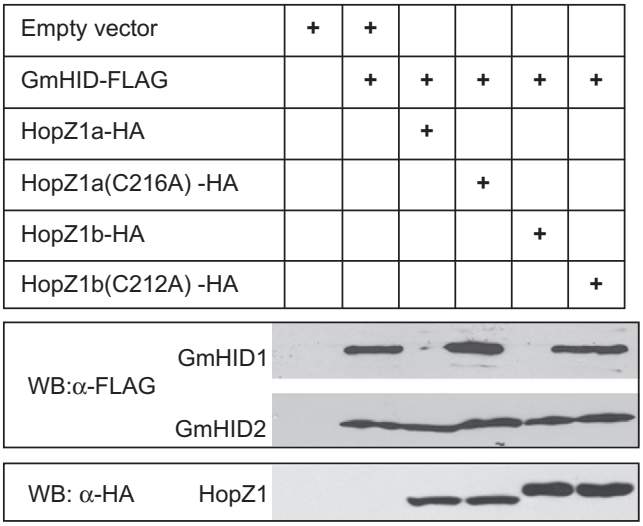

Figure 2. HopZ1 Triggers GmHID1 Degradation In Planta

GmHID1-FLAG was coexpressed in *N. benthamiana* leaves with HopZ1a-HA, HopZ1a(C216A)-HA, HopZ1b-HA, or HopZ1b(C212A)-HA using *Agrobacterium*-mediated transient expression. Leaf tissues were collected 24 hr postinfiltration, and the total proteins were analyzed by western blots using anti-FLAG antibody to detect GmHID1-FLAG and anti-HA antibody to detect HopZ1-HA. The experiment was repeated using GmHID2-FLAG in replacement of GmHID1-FLAG in order to verify the specific degradation of GmHID1 by HopZ1. This experiment was repeated five times with the same results. (See also Figure S2.)

nonfluorescent N-terminal domain of YFP (1–155 aa, nYFP) and the C-terminal domain of YFP (156 239 aa, cYFP) at their C termini, respectively. The fusion genes were transiently expressed in *N. benthamiana*, and the presence of yellow fluorescence in the pavement cells was examined using confocal microscopy. We observed no fluorescence in the infiltrated leaves at 24 hpi, which was apparently too early to detect fluorescence in BiFC assays. Since HopZ1a and HopZ1b both induce HR in *N. benthamiana* in later time points, we repeated the experiment using the catalytic mutants HopZ1a(C216A) and HopZ1b(C212A), which do not trigger HR (Ma et al., 2006; Zhou et al., 2009). These catalytic mutants are not altered in their interaction with GmHID1 and the subcellular localization in plant cells (Figure 1A and data not shown). At 48 hpi, yellow fluorescence was observed in cells expressing GmHID1-cYFP together with either HopZ1a(C216A)-nYFP or HopZ1b(C212A)-nYFP, but not in the cells expressing GmHID1-cYFP + nYFP or GmHID2-cYFP + HopZ1(C/A)-nYFP (Figure 1D). The fluorescence was mainly present on the plasma membrane and also in the nucleus (Figure 1D), which is consistent with the localization of HopZ1 and GmHID1 in planta.

Interaction with HopZ1 Leads to the Degradation of GmHID1

Since HopZ1 proteins are predicted to have potential cysteine protease or acetyltransferase activities, we next examined whether GmHID1 was modified upon interaction with HopZ1. FLAG-tagged GmHID1 was coexpressed in *N. benthamiana* with HopZ1-HA using *Agrobacterium*-mediated transient expression. Total proteins were extracted from the infiltrated leaf tissue at 24 hpi, and GmHID1-FLAG was examined by western blots using anti-FLAG antibody. We observed a significant reduction of GmHID1-FLAG proteins in *N. benthamiana* leaves coexpressing HopZ1a-HA or HopZ1b-HA compared to the leaves expressing GmHID1-FLAG and the empty vector (Figure 2). This reduction of GmHID1-FLAG proteins was not observed in leaves coexpressing GmHID1-FLAG and the catalytic mutants HopZ1a(C216A)-HA or HopZ1b(C212A)-HA (Figure 2). These results demonstrated that HopZ1a and HopZ1b induced the degradation of GmHID1 proteins in planta; and the degradation was dependent on the enzymatic activity of HopZ1.

To exclude the possibility that the reduction in the amount of GmHID1 proteins simply resulted from the programmed cell death triggered by HopZ1a and HopZ1b in *N. benthamiana*, we coexpressed GmHID2-FLAG with HopZ1-HA or HopZ1(C/A)-HA in *N. benthamiana*. The protein levels of GmHID2-FLAG remained the same in the presence of either the wild-type or the catalytic mutants of HopZ1-HA (Figure 2). We further strengthen this conclusion by coexpressing HopZ1a-HA and GmHID1-FLAG in the protoplasts of the *Arabidopsis thaliana zar1* mutant, which is unable to elicit HR responding to HopZ1a (Lewis et al., 2010). Again, a significant reduction of GmHID1 was observed when it was coexpressed with HopZ1a in the protoplasts (Figure S2). These data suggest that GmHID1 is a substrate of HopZ1, which induces GmHID1 degradation in planta.

gmhid1 Gene Expression Is Upregulated upon *P. syringae* Infection

We next determined the gene expression pattern of *gmhid1* in soybean leaf tissues during *P. syringae* infections. The transcripts of *gmhid1* were monitored in soybean unifoliates over a period of 0–72 hr after inoculated with *Pgy* strains (OD_{600} = 0.0001). *gmhid1* has a low basal expression level in soybean leaves without bacterial infection (Figure 3A). However, it was induced and reached the maximum expression level at around 12 hpi in the leaves infected with *Pgy*BR1Rif (Figure 3A). The expression of *gmhid1* was induced to a similar level in the soybean leaves infected with *Pgy*BR1Rif-O1 (Figure 3B), a mutant of *Pgy*BR1Rif with the endogenous plasmid carrying *hopZ1b* cured, suggesting that *Pgy*-induced *gmhid1* expression was independent of the presence of HopZ1b. Furthermore, in leaves inoculated with a T3SS knockout mutant, *Pgy*BR1-Rif::*hrcJΩnptII*, *gmhid1* expression was induced, but to a much lower level compared to the leaves infected with *Pgy*BR1 or *Pgy*BR1-O1 (Figure 3B). These data indicate that the induction of the *gmhid1* expression was largely reinforced by the presence of the T3SS.

HopZ1 Suppresses Daidzein Biosynthesis in Soybean Infected with *P. syringae*

GmHID1 catalyzes the enzymatic reaction to produce genistein and daidzein in soybean. To examine the impact of HopZ1 on isoflavone biosynthesis, the amount of daidzein was measured in soybean leaf tissues infected with *Pgy*. Isoflavonoids were extracted from the infected unifoliates at 12 hpi using a protocol described previously (Lozovaya et al., 2004). After separation by HPLC, the absorbance peak representing daidzein was identified using mass spectrometry, and the quantity of daidzein

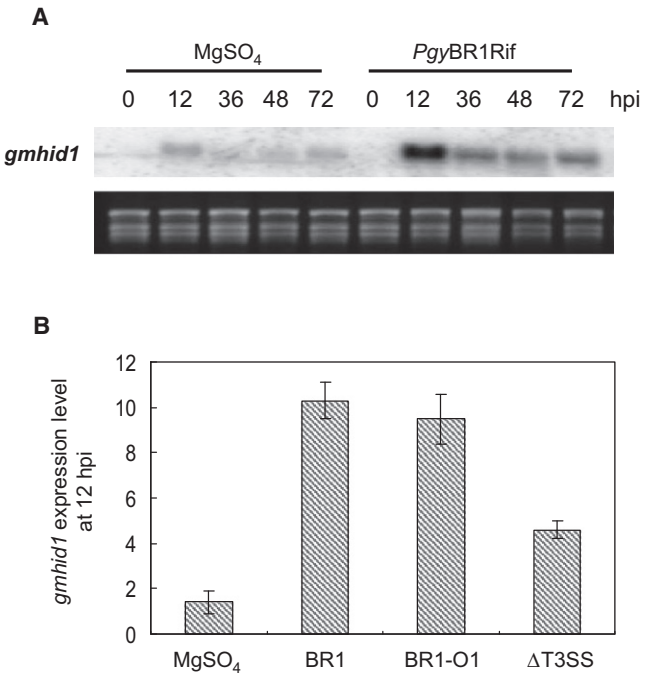

Figure 3. *gmhid1* Induction in Soybean Leaves upon Infection of *P. syringae* pv. glycinea

Unifoliates of 10-day soybean seedlings were inoculated with *P. syringae* pv. glycinea (*Pgy*) strains at OD_{600} = 0.0001. Total RNA was extracted from the infected leaves, and *gmhid1* transcripts were analyzed by northern blots (A) or real-time RT-PCR (B). Leaves infiltrated with 10 mM $MgSO_4$ was used as the mock treatment control.

(A) *gmhid1* is induced in soybean inoculated with *Pgy*BR1Rif. *gmhid1* transcripts were examined during a period of 0–72 hr postinfection by northern blots using a gene-specific probe.

(B) *gmhid1* induction by different *Pgy* strains. RNA samples were extracted from soybean leaves infected with BR1Rif (BR1), BR1Rif-O1 (BR1-O1), or BR1Rif::*hrcJΩnptII* (ΔT3SS) at 12 hpi. Real-time RT-PCR was performed using primers amplifying *gmhid1* and *gmubi* (ubiquitin) genes. The number of *gmhid1* transcripts was normalized using the number of *gmubi* transcripts from the same sample. *gmhid1* induction, represented as fold difference, was determined by comparing the normalized *gmhid1* transcripts from leaf tissues infected with *P. syringae* to that from the mock treatment control. The experiments were repeated three times with similar results. Data from one representative experiment are presented with error bars representing the standard deviations from three replicates.

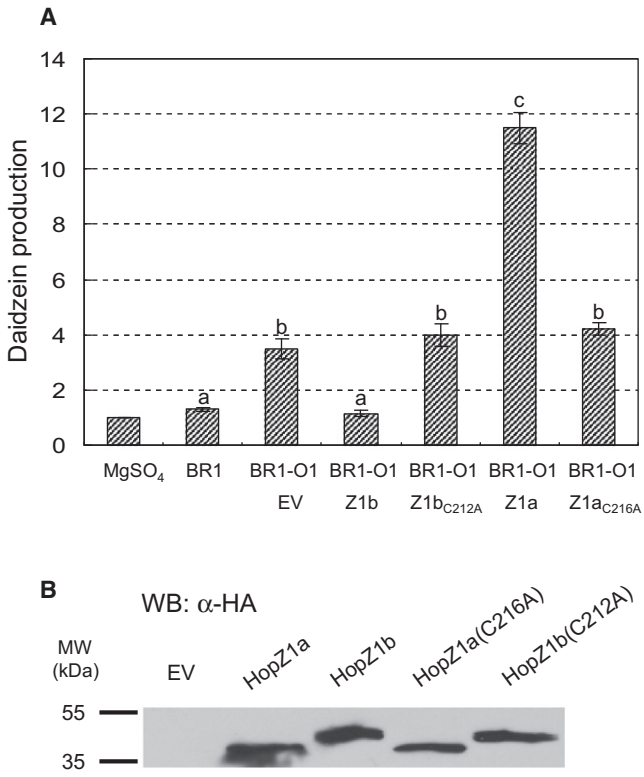

Figure 4. HopZ1b Reduces Daidzein Production in Soybean Leaves Infected with *P. syringae* pv. glycinea

(A) Daidzein production in soybean leaves infected with *Pgy* strains. Unifoliates of 10-day soybean seedlings were inoculated with *Pgy* strains BR1Rif (BR1), BR1Rif-O1(pUCP20tk) (BR1-O1/EV), BR1Rif-O1(pUCP20tk::HopZ1a-HA) (BR1-O1/Z1a), BR1Rif-O1(pUCP20tk::HopZ1a(C216A)-HA) (BR1-O1/$Z1a_{C216A}$), BR1Rif-O1(pUCP20tk::HopZ1b-HA) (BR1-O1/Z1b), or BR1Rif-O1 (pUCP20tk::HopZ1b(C212A)-HA) (BR1-O1/$Z1b_{C212A}$) at OD_{600} = 0.001. Leaves infiltrated with 10 mM $MgSO_4$ were used as the mock treatment control. Daidzein was extracted from the infected leaves at 12 hpi and analyzed by HPLC. Fold changes of the amount of daidzein produced in the infected leaves compared to the mock treatment leaves are presented. This experiment was repeated four times with similar results. Data are represented as the means and standard deviations (as error bars) generated from three biological replicates of one representative experiment. Different letters indicate statistically significant differences between the samples.

(B) Immunoblot showing the protein expression of HopZ1 in *Pgy*BR1Rif-O1. *Pgy*BR1Rif-O1 cells expressing HopZ1-HA were induced in M63 minimal medium containing 1% fructose. Total bacterial proteins from equal amount of induced cells were extracted and HopZ1 proteins were detected by western blot using anti-HA antibody.

was estimated. In leaves infected with *Pgy*BR1Rif-O1, which does not produce HopZ1b, the amount of daidzein was increased approximately 3.5-fold compared to the mock-infected control (Figure 4A). However, soybean leaves infected with *Pgy*BR1Rif, carrying an endogenous *hopZ1b*, produced a significantly lower amount of daidzein, comparable to that in the mock treatment plants (Figure 4A). These data indicate that HopZ1b produced and delivered by *Pgy*BR1Rif to soybean cells may have inhibited daidzein production.

To further confirm that HopZ1b was responsible for the reduction of daidzein in *Pgy*BR1Rif-infected leaves, daidzein was measured in soybean leaves inoculated with *Pgy*BR1Rif-O1 carrying the plasmids pUCP20tk::HopZ1b-HA or pUCP20tk::HopZ1b(C212A)-HA. Again, leaves infected with *Pgy*BR1Rif-O1 expressing HopZ1b-HA produced a much lower amount of daidzein compared to the leaves infected with *Pgy*BR1Rif-O1 expressing HopZ1b(C212A)-HA or the leaves infected with *Pgy*BR1Rif-O1 carrying the empty vector pUCP20tk (Figure 4A). Since the catalytic mutant HopZ1b(C212A) interacts with GmHID1 in the same way as the wild-type HopZ1b, these data demonstrated that the enzymatic activity of HopZ1b was required for the inhibition of daidzein production. A 10- to 12-fold increase in daidzein production was observed in the leaves infected with *Pgy*BR1Rif-O1 expressing HopZ1a-HA (Figure 4A). This strong induction of daidzein production is likely associated with the HR triggered by HopZ1a in soybean.

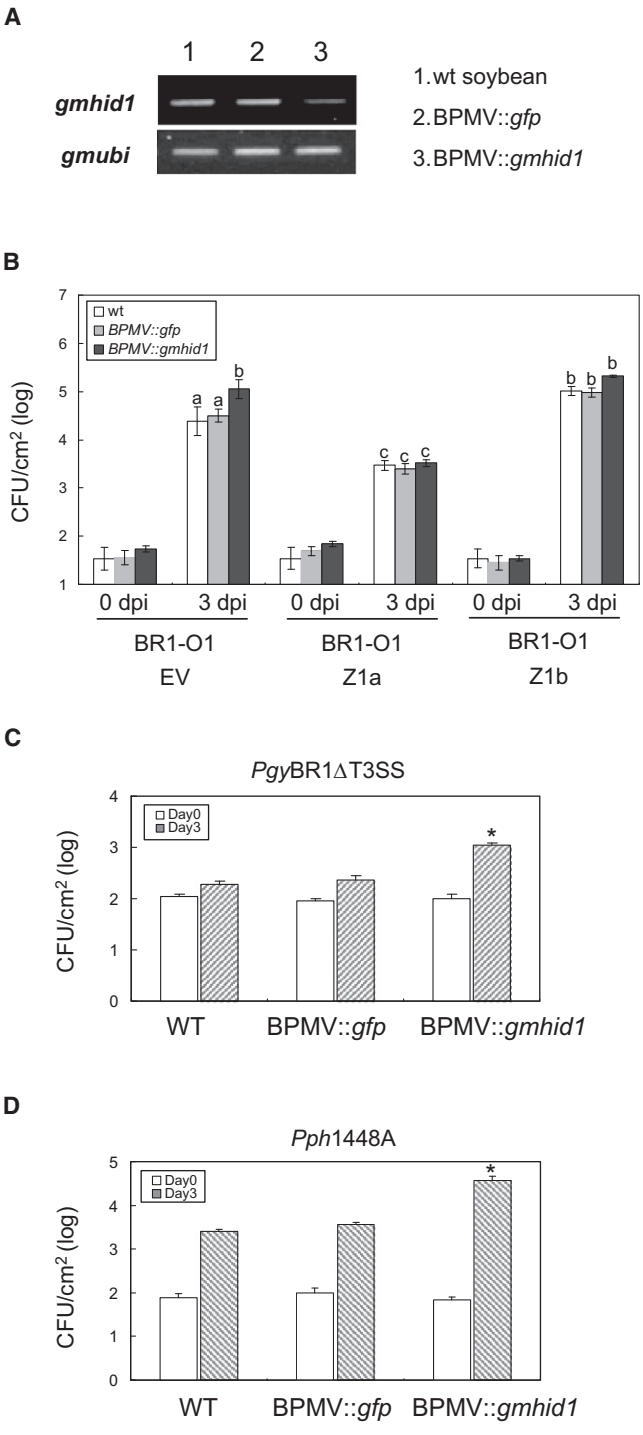

Figure 5. Silencing of *gmhid1* Leads to Increased Susceptibility of Soybean to *P. syringae* Infection

In planta bacterial multiplication assays were performed using the wild-type soybean cv. Williams or soybean plants inoculated with BPMV::*gfp* or BPMV::*gmhid1*. After the establishment of gene silencing, the third or fourth trifoliates were infiltrated with bacterial suspensions at OD_{600} = 0.0001 (*Pgy*) and OD_{600} = 0.0005 (*Pph*1448A). Bacterial populations were evaluated at 0 and 3 dpi. Cfus were counted in leaf disks punched from three different plants. The average cfu and standard deviations (as error bars) using data from one representative experiment are presented. Different letters or asterisks indicate statistically significant differences between the samples. All experiments were repeated five times with similar results.

(A) Semiquantitative RT-PCR showing the reduced accumulation of *gmhid1* transcripts in BPMV::*gmhid1*-inoculated soybean plants.

(B) In planta multiplications of *Pgy* strains BR1Rif-O1(pUCP20tk) (BR1-O1/EV), BR1Rif-O1(pUCP20tk::HopZ1a-HA) (BR1-O1/Z1a), and BR1Rif-O1(pUCP20tk::HopZ1b-HA) (BR1-O1/Z1b).

(C) In planta multiplication of *Pgy* strain BR1Rif::*hrcJΩnptII* (*Pgy*BR1ΔT3SS).

(D) In planta multiplication of *P. syringae* pv. phaseolicola strain 1448A (*Pph*1448A).

The protein expression of HopZ1-HA in *Pgy*BR1Rif-O1 was verified by western blots (Figure 4B), confirming that the difference in the amounts of daidzein was not due to different protein expression levels of HopZ1 proteins in *Pgy*BR1Rif-O1.

Silencing *gmhid1* Expression Increases the Susceptibility of Soybean to *P. syringae* Infection

The contribution of GmHID1 to soybean resistance against *P. syringae* infection was examined by virus-induced gene silencing (VIGS) using a bean pod mottle virus (BPMV)-based vector (Zhang and Ghabrial, 2006). Viral RNA containing a partial *gmhid1* gene sequence was in vitro transcribed and used to mechanically inoculate the unifoliates of soybean seedlings. The third or fourth trifoliates of the inoculated plants with confirmed silencing of *gmhid1* gene (Figure 5A) were used for bacterial multiplication assays. BPMV::*gfp*-inoculated plants were used as controls to confirm that BPMV did not interfere with the multiplication of *P. syringae* in soybean.

Pgy strains *Pgy*BR1Rif-O1 carrying the empty vector pUCP20tk, pUCP20tk::HopZ1b-HA, or pUCP20tk::HopZ1b (C212A)-HA were used to inoculate the wild-type, BPMV::*gfp*-, and BPMV::*GmHID1*-infected soybean plants. Bacterial populations were measured 3 days postinoculation (dpi). In wild-type soybean, *Pgy*BR1Rif-O1 carrying pUCP20tk::HopZ1b-HA multiplied to a significantly higher level than did *Pgy*BR1Rif-O1 carrying the empty vector pUCP20tk or pUCP20tk::HopZ1b (C212A)-HA, indicating that HopZ1b promotes bacterial multiplication in soybean. In *gmhid1*-silenced soybeans, the bacterial population of *Pgy*BR1Rif-O1 carrying the empty vector pUCP20tk was approximately 5-fold higher than in the wild-type soybean or the BPMV::*gfp* plants (Figure 5B). Moreover, the population of *Pgy*BR1Rif-O1 carrying the empty vector pUCP20tk in the *gmhid1*-silenced soybeans was very similar to that of *Pgy*BR1Rif-O1 carrying pUCP20tk::HopZ1b-HA in the wild-type soybean (Figure 5B), indicating that the growth-promoting activity of HopZ1b in soybean was mostly compromised by the silencing of *gmhid1*.

In order to examine the potential function of GmHID1 in soybean basal defense, in planta populations of *Pgy*BR1Rif-O1*hrcJΩnptII* and *P. syringae* pv. phaseolicola strain 1448A (*Pph*1448A) were determined. *Pgy*BR1Rif-O1*hrcJΩnptII*, without a functional T3SS, is unable to cause disease or multiply in soybean (Zhou et al., 2009). *Pph*1448A, originally isolated from common beans, multiplies poorly in soybean. In the wild-type soybean or the BPMV::*gfp*-infected plants, populations of *Pgy*BR1Rif-O1*hrcJΩnptII* and *Pph*1448A remained low at 3 dpi (Figures 5C and 5D). However, an approximately 10-fold increase in bacterial populations of these two bacteria was observed in the *gmhid1*-silenced soybean plants (Figures 5C

and 5D). These data suggest that GmHID1 plays a role in restricting *P. syringae* multiplication in soybean.

Since HopZ1a triggers HR in soybean, *Pgy*BR1Rif-O1 carrying pUCP20tk::HopZ1a-HA multiplied to a much lower level than did *Pgy*BR1Rif-O1 carrying the empty vector pUCP20tk in soybean (Figure 5B). The populations of *Pgy*BR1Rif-O1 carrying pUCP20tk::HopZ1a-HA were similar in the *gmhid1*-silenced, the BPMV::*gfp*-infected, and the wild-type soybean plants (Figure 5B), demonstrating that HopZ1a triggered HR in the presence or absence of GmHID1. These data suggest that interaction with (and degradation of) GmHID1 is not responsible for the HR-triggering activity of HopZ1a. This is consistent with our previous hypothesis that HopZ1a-specific substrates determine its recognition in soybean (Morgan et al., 2010). Since GmHID1 interacts with both HopZ1a and HopZ1b, it is unlikely that GmHID1 is involved in HopZ1a-triggered HR in soybean.

DISCUSSION

T3SEs target specific host substrates and promote bacterial virulence. So far, our knowledge of the molecular mechanisms underlying the functionality of T3SEs is limited. Particularly, very few direct virulence targets of T3SEs have been identified, and most of these reported targets are primarily involved in defense signaling transduction. In this study, we show that HopZ1 promotes *P. syringae* multiplication in soybean by targeting the enzyme 2-hydroxyisoflavanone dehydratase (GmHID1), which is involved in isoflavonoid biosynthesis. *gmhid1* is induced upon *P. syringae* infection; and silencing of *gmhid1* transcription in soybean leads to enhanced susceptibility to *P. syringae* infection, suggesting a role of this enzyme in basal defense. Two HopZ1 alleles, HopZ1a and HopZ1b, directly interact with GmHID1 and induce the degradation of GmHID1 in plant cells. Furthermore, HopZ1b produced by the soybean pathogen *Pgy* can reduce the production of daidzein in *Pgy*-infected soybean leaves. These data support HopZ1 as an example of T3SEs that directly target a biosynthetic enzyme in host secondary metabolism to suppress plant defense.

Responsible for catalyzing the conversion of 2-hydroxyisoflavanone to isoflavones, GmHID1 plays an important role in isoflavonoid production in soybean (Akashi et al., 2005; Shimamura et al., 2007). This was evidenced by the significant increase of daidzein and genistein production in *Lotus japonicus* hairy roots when GmHID1 was overexpressed (Shimamura et al., 2007). Although dehydration of 2-hydroxyisoflavanone can occur spontaneously to produce isoflavones, this spontaneous nonenzymatic process was much slower than the enzyme-catalyzed reaction (Akashi et al., 1999). It has been proposed that the spontaneous dehydration of 2-hydroxyisoflavanone may be an alternative process when rapid production of isoflavones is not necessary (Akashi et al., 2005). Therefore, the activity of GmHID1 can be particularly important in *P. syringae*-infected tissues where the production of isoflavonoids is rapidly induced, presumably as a defense response.

An approximately 11-fold increase of daidzein was observed in the soybean leaves inoculated with *Pgy*BR1-O1 producing HopZ1a. This strong induction of daidzein production is likely in association with the HR triggered by HopZ1a. Interestingly, a relatively moderate (3-to 4-fold) daidzein induction was found in soybean leaves infected with the *hopZ1b* knockout mutant (*Pgy*BR1-O1) of the compatible pathogen *Pgy*BR1. This moderate daidzein induction might be associated with a role in basal defense. Daidzein is the immediate precursor of glyceollins, the major phytoalexins in soybean (Lozovaya et al., 2007). Phytoalexins are believed to act as antimicrobial compounds (Bednarek and Osbourn, 2009), but direct evidence of isoflavones or phytoalexins functioning in defending plants against bacterial infections, especially during the compatible interactions, has not been reported. Our preliminary studies did not find any direct inhibitory effect of daidzein on *P. syringae* growth in vitro (data not shown). Future work will be required to understand whether daidzein and/or glyceollins are involved in PTI during bacterial infection. A particularly interesting possibility is that these compounds might be involved in defense signaling.

Consistent with the induction of daidzein in *P. syringae*-infected soybean leaves, the *gmhid1* gene was induced upon *Pgy* infection. A T3SS mutant was still able to induce *gmhid1* expression, indicating that *gmhid1* induction could be triggered by PAMPs. However, the induction of *gmhid1* was largely reinforced in the presence of the T3SS, and this reinforcement was independent of HopZ1b. It is possible that some of the other T3SEs delivered from *Pgy* can trigger weak plant defense responses which induce *gmhid1* expression. Another intriguing possibility would be that *gmhid1* expression was induced by structural components of T3SS. Animal hosts could detect the T3SS basal body rod from bacterial pathogens and trigger innate immunity (Miao et al., 2010). Recently, the T3SS translocator HrpK1 of *P. syringae* pv. tomato DC3000 was reported to elicit reactive oxygen species (ROS) accumulation in *N. benthamiana* (Oh et al., 2010). It is therefore possible that, similar to animal systems, the T3SS structural components can also trigger plant immunity, such as the isoflavonoid productions in soybean.

YopJ-like effectors contain the canonical catalytic triad (H-E-C) identical to that of clan CE (C55 family) cysteine proteases. However, YopJ has been demonstrated to be an acetyltransferase in animal cells (Mittal et al., 2006; Mukherjee et al., 2006). Although HopZ1 induces the degradation of GmHID1 in planta, we cannot exclude the possibility that the degradation of GmHID1 is indirect, after its acetylation by HopZ1. To test whether HopZ1 possesses an acetyltransferase activity, in vitro acetylation assays were conducted following a previously described procedure (Mukherjee et al., 2006). Using C14-labeled acetyl-CoA and recombinant GmHID1 and HopZ1 proteins purified from *E. coli*, no acetyltransferase activity of HopZ1 was detected despite numerous trials (data not shown). A recent study also failed to detect an acetyltransferase activity from another YopJ-like T3SE, AvrBsT, using in vitro assays (Szczesny et al., 2010). HopZ1a and AvrBsT have weak cysteine protease activities in vitro (Ma et al., 2006; Szczesny et al., 2010). However, incubation of the recombinant GmHID1 and HopZ1 proteins purified from *E. coli* did not cause an observable degradation of GmHID1 (Figure 2B). Although we cannot rule out the possibility that the buffer conditions used in these in vitro assays were not suitable for HopZ1, it is more likely that HopZ1 requires some unknown cofactor(s) or activator(s) for its yet-to-be-determined enzymatic activity in plant cells.

While the enzymatic activities of YopJ-like T3SEs in plant pathogens remain a dilemma, currently available evidence suggests

that these YopJ-like T3SEs may possess different enzymatic activities and target different host substrates. Phylogenic analysis shows that YopJ-like T3SEs can be divided into two major clades: one clade includes the majority of the YopJ homologs from plant pathogens; the other clade includes all the YopJ homologs from animal pathogens (Figure S3), suggesting that the YopJ-like T3SEs in plant pathogens and animal pathogens have diverged and evolved independently afterwards. The YopJ-like T3SEs in plant pathogens can be further divided into two main subgroups with PopP2 from *Ralstonia solanacearum* distantly related to both groups. This further divergence of YopJ-like T3SEs in plant pathogens, which is not as evident in the YopJ homologs in animal pathogens, may explain why several different enzymatic activities have been observed in YopJ-like T3SEs in plant pathogens. The divergence in the cellular function of YopJ-like T3SEs in plant pathogens is also reflected by the diverse protein localizations of these proteins in planta. For example, AvrRxv is mainly localized to the cytoplasm (Bonshtien et al., 2005); XopJ is anchored to the plasma membrane and also localized to vesicle-like structures (Bartetzko et al., 2009; Thieme et al., 2007); and AvrBsT localizes to both cytoplasm and nucleus (Szczesny et al., 2010). HopZ1, mainly located on the plasma membrane, is soluble and present in both cytoplasm and nucleus (Figure 1C). These pieces of evidence suggest that YopJ-like effectors have evolved to target different host substrates in adaptations to their specific host environments.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids

Pseudomonas syringae, *Agrobacterium tumefaciens*, and *Escherichia coli* strains were grown as described in Morgan et al. (2010). Bacteria strains and plasmids used in this study are summarized in Table S1.

Yeast Two-Hybrid Screens

HopZ1 alleles were cloned into the yeast vector pGBKT7 (Clontech). The expression of the BD-HopZ1 fusion proteins has been confirmed by western blots (data not shown). A soybean (*Glycine max* [L.] cv. William 82) cDNA library was constructed in the vector pGADT7-RecAB using total RNA extracted from soybean leaf tissues collected 8 and 24 hr after the plants were spray-inoculated with *P. syringae* pv. glycinea strain BR1Rif (*Pgy*BR1Rif) or *Pgy*BR1Rif carrying the plasmid pUCP20tk::HopZ1a-HA at OD_{600} = 0.0001 (approximately 2 × 10^5 cfu/mL). Approximately 1.2 × 10^7 primary yeast clones (four times coverage of the library) were screened using HopZ1a and HopZ1b as the baits. Potential yeast transformants containing cDNA clones interacting with HopZ1 were selected using the selective minimal synthetic defined (SD) medium lacking adenine, tryptophan, histidine, and leucine. The positive clones were also verified by a blue/white color selection using the same SD medium supplemented with X-α-gal.

Fluorescence Microscopy

To construct plasmids for BiFC assay, full-length cDNA of *gmhid1* or *gmhid2* fused to the C terminus of YFP and *hopZ1(C/A)* fused to the N terminus of YFP were cloned into the vectors pSPYCE and pSPYNE (Walter et al., 2004), respectively. For constructs for examining the subcellular localization of GmHID1 and HopZ1, full-length cDNAs were cloned into the vector pEG101 (Earley et al., 2006). The recombinant plasmids were introduced into *Agrobacterium tumefaciens* strain C58C1(pCH32). Leaves of 3-week-old *Nicotiana benthamiana* plants were infiltrated with *Agrobacterium* expressing GmHID1-YFP or HopZ1-YFP or coinfiltrated with *Agrobacterium* expressing GmHID-cYFP and HopZ1(C/A)-nYFP using a protocol described previously (Zhou et al., 2009). Functional fluorophore were visualized in the infiltrated leaves using a Leica SP2 Laser Scanning Confocal Microscope (Leica Microsystems) at 24 hr postinfection for subcellular localization or 48 hr postinfection for BiFC.

In Vitro GST Pull-Down Assays

To construct GST-fusion plasmids, *hopZ1a* and *hopZ1b* were inserted into the vector pGEX4T-2 (GE Healthcare Life Science). To construct FLAG-fusion plasmid, *gmhid1-FLAG* was inserted into the vector pET14b (Navogen). Pull-down assay was carried out using ProFound pull-down GST protein:protein interaction kit (Pierce) according to the manufacturer's instructions. Briefly, GST or GST-HopZ1 was expressed in *E. coli* strain BL21(DE3). Soluble proteins were incubated with 50 μl glutathione agarose beads (Invitrogen) for 1 hr at 4°C. The beads were washed five times and then incubated with an equal amount of bacterial lysates containing GmHID1-FLAG for another hour at 4°C. The beads were washed five times again, and the presence of GmHID1-FLAG was detected by immunoblotting using anti-FLAG antibody conjugated with horseradish peroxidase (HRP) (Santa Cruz Biotechnology Inc.).

Real-Time RT-PCR

The expression of *gmhid1* gene in soybean leaf tissues was analyzed by real-time RT-PCR using the DNA binding dye SYBR Green I and an iQ5 Real-Time PCR Detection System (BioRad Laboratories) following the manufacturer's instructions. Total RNA was isolated from three independent biological replicates using a hot phenol extraction method (Salter and Conlon, 2007). DNA was removed from samples using DNase I (Fermentas). Reverse transcription was performed using M-MLV Reverse Transcriptase (Promega) with 1 μg of total RNA in a total reaction of 25 μL. The cDNAs were then used as templates for real-time PCR using gene-specific primers for *gmhid1* and *gmubi* (as an internal control). Primers 5′-GGCGAAGGAGATAGTGAAAGAGC-3′ and 5′-GTGTGGTGGGATTTGGGAAGG-3′ were used for *gmhid1*; and primers 5′-AGATTACGAAACCGCCAACTACC-3′ and 5′-GGAAGGAGGAGTGGGTG TAGG-3′ were used for *gmubi*. The number of *gmhid1* transcripts was normalized using the number of *gmubi* transcripts from the same sample. *gmhid1* induction was determined by comparing the normalized *gmhid1* transcripts from the leaf tissues infected with *P. syringae* to that from the leaves infiltrated with 10 mM $MgSO_4$ (the mock treatment).

HPLC Analysis

Soybean leaf tissues were ground in liquid nitrogen and extracted with ten volumes of 80% methanol (v/v) at 4°C with gentle shaking for 12 hr. The supernatant was filtered through 0.45 μm filters, and a 5 μL sample was separated by HPLC (Waters 2698 separation modules) with a Kromasil 5 μm C18 column (Peeke Scientific) using a gradient solvent system (0.2% acetic acid and acetonitrile). After sample injection, acetonitrile was started from 8% to 40% v/v during the first 8 min and then increased to 90% in the following 10 min and kept at 90% at the last 10 min. The solvent flow rate was 0.3 mL/min. A Waters 486 Tunable Absorbance detector with a 280 nm wavelength was used for detection. The amount of daidzein was estimated by measuring the area of the peak generated by daidzein in chromatograph. A daidzein standard was purchased from Sigma Aldrich.

Soybean In Planta Bacterial Multiplication Assays

Soybeans were planted as previous described (Zhou et al., 2009). The unifoliates of 10-day-old soybean seedlings were infiltrated with the bacterial cell suspensions using a needleless syringe. The cells were suspended in 10 mM $MgSO_4$ at an OD_{600} = 0.0001 for *Pgy* strains and OD_{600} = 0.0005 for *Pph*1448A. The inoculated plants were transferred to a growth chamber (22°C and 16/8 hr light/dark regime). To determine the in planta bacteria populations, four leaf discs (0.2 cm^2) were taken from each inoculated leaf at 0 and 3 days postinoculation. Bacterial populations were determined as colony-forming units (cfus) per cm^2 using a previously described procedure (Morgan et al., 2010).

Virus-Induced Gene Silencing of Soybean

Plasmids pGHoR1, pGG7R2::*gfp*, and pGG7R2::*gmhid1* were linearized and used as templates for in vitro transcription as described by Zhang and Ghabrial (2006). A mixture of RNA1 (transcribed from pGHoR1) and RNA2 transcripts (transcribed from either pGG7R2::*gfp* or pGG7R2-*gmhid1*) was used to inoculate the unifoliates of 10-day-old soybean seedlings by rubbing. The

inoculated plants were grown in a growth chamber (22°C, 16/8 hr light/dark regime) for about 3–4 weeks until mild virus infection symptoms appeared on the third and fourth trifoliates. Silencing of *gmhid1* was verified by semi-quantitative RT-PCR using one of the trifoliates. The other two leaves of the tested trifoliates were then used for in planta bacterial multiplication assays. Due to the high sequence similarity between *gmhid1* and *gmhid2*, our constructs silenced both genes.

Protein Analysis

P. syringae expressing the *hopZ1* genes carried on pUCP20tk was induced in M63 minimal medium containing 1% fructose at room temperature overnight (Morgan et al., 2010). HopZ1 expression was detected by western blots using anti-HA antibody.

Four-week-old *N. benthamiana* leaves were infiltrated with *A. tumefaciens* strain C58C1 (pCH32) harboring binary vectors carrying the *hopZ1* and/or *gmhid* genes at OD_{600} = 0.5 as previously described (Ma et al., 2006; Szurek et al., 2001). Inoculated plants were kept at room temperature under continuous low light. Leaf disks were grounded in 2 × Laemmli buffer and the presence of GmHID-FLAG and HopZ1-HA was analyzed by western blots using anti-FLAG and anti-HA antibodies, respectively.

Statistical Analysis

Statistical analyses were performed using JMP 8.0 (SAS Institute Inc.).

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and one table and can be found with this article at doi:10.1016/j.chom.2011.02.007.

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