

# The Conjugated Auxin Indole-3-Acetic Acid–Aspartic Acid Promotes Plant Disease Development

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**Auxin is a pivotal plant hormone that regulates many aspects of plant growth and development. Auxin signaling is also known to promote plant disease caused by plant pathogens. However, the mechanism by which this hormone confers susceptibility to pathogens is not well understood. Here, we present evidence that fungal and bacterial plant pathogens hijack the host auxin metabolism in *Arabidopsis thaliana*, leading to the accumulation of a conjugated form of the hormone, indole-3-acetic acid (IAA)–Asp, to promote disease development. We also show that IAA–Asp increases pathogen progression in the plant by regulating the transcription of virulence genes. These data highlight a novel mechanism to promote plant susceptibility to pathogens through auxin conjugation.**

## INTRODUCTION

Plants fight microbial attacks using both constitutive and induced defenses, which include basal and highly specific resistance (Jones and Dangl, 2006). Basal resistance (PAMP-triggered immunity [PTI]) is often mediated via the detection of pathogen-associated molecular patterns (PAMPs). PAMPs include molecules that are associated with several classes of pathogens, such as polysaccharides and bacterial flagellin (Jones and Dangl, 2006; Zipfel, 2009). However, adapted microbes express a suite of effector proteins that often act to suppress these defenses (Dodds and Rathjen, 2010). Plants have evolved other receptors (resistance proteins) that detect these pathogen effectors and activate strong defenses called effector-triggered immunity (Jones and Dangl, 2006; Zipfel, 2009). The plant hormones salicylic acid (SA) and jasmonic acid (JA) are major players in the regulation of signaling networks that are involved in PTI and effector-triggered immunity (Jones and Dangl, 2006; van Loon et al., 2006; Bent and Mackey, 2007; Zipfel, 2009). In general, SA is required against biotrophic pathogens that benefit from a live host cell, while JA is effective against necrotrophs that benefit from host cell death (Grant and Lamb, 2006).


Auxins constitute a small group of plant hormones that are involved in the regulation of numerous aspects of plant growth and development (Santner and Estelle, 2009). By far the most prominent and best studied auxin is indole-3-acetic acid (IAA), which has been linked to such diverse processes as elongation


and division of cells, tropic responses, vascular development, response to biotic and abiotic stimuli, and the general organization of root and shoot architecture (Santner and Estelle, 2009; Kieffer et al., 2010). The amount of active IAA in specific tissues is tightly regulated and determined by an array of metabolic processes, including regulation of its synthesis, transport to or from specific cells or tissues, IAA inactivation and reactivation, and degradation via multiple oxidative pathways (Normanly, 2010). The last two processes involve IAA modification to glycosyl esters and amide-linked (with various amino acids and peptides) conjugates. In *Arabidopsis thaliana*, the majority of the conjugated forms are linked to amino acids or small peptides. IAA–amino acid conjugates are classified as storage and catabolism conjugates (Ludwig-Müller, 2011). The storage conjugates, including IAA–Ala and IAA–Leu, can be hydrolyzed by plant enzymes to give free auxin, while the catabolism conjugates, such as IAA–Asp and IAA–Glu, are not a substrate of IAA–amino acid aminohydrolases. Since free IAA is established as being the biologically active form of auxin, only certain hydrolysable conjugates have been shown to be active in auxin bioassays (Woodward and Bartel, 2005; Ludwig-Müller, 2011). Although a role for IAA–Asp in abiotic stress and ripening has been suggested in henbane (*Hyoscyamus niger*) and grape (*Vitis vinifera*) (Oetiker and Aeschbacher, 1997; Böttcher et al., 2010), a direct biological function for IAA–Asp and IAA–Glu has not been clearly demonstrated, and these conjugates are rather considered as the starting point of auxin catabolism (Woodward and Bartel, 2005; Ludwig-Müller, 2011). Auxin conjugation to amino acids is carried out by the family of GH3 proteins. This gene family consists of 19 members, of which at least seven were shown to catalyze the synthesis of IAA–amino acid conjugates (Staswick et al., 2005).

A role for auxin in plant disease has long been suspected since many pathogens produce auxin during interaction with their host and because auxin can modulate the plant defense response (Yamada, 1993; Yang et al., 2007; Kazan and Manners, 2009). Auxin signaling has also been shown to promote disease caused by a number of pathogens (Grant and Jones, 2009; Kazan and

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Manners, 2009), and several auxin signaling mutants are more resistant to necrotrophic and biotrophic pathogens than are wild-type plants (Navarro et al., 2006; Chen et al., 2007; El Oirdi and Bouarab, 2007; Wang et al., 2007; Cuzick et al., 2009; Kidd et al., 2011). However, the mechanism by which auxin signaling promotes disease is not yet understood. One of the responses induced by the activation of auxin signaling is the formation of the conjugated forms of auxin through the action of GH3 proteins. Interestingly, recent works have shown the involvement of some members of the GH3 gene family in the plant–pathogen interaction in *Arabidopsis* and rice (*Oryza sativa*) (Jagadeeswaran et al., 2007; Nobuta et al., 2007; Park et al., 2007; Zhang et al., 2007; Ding et al., 2008; Domingo et al., 2009; Kazan and Manners, 2009; Fu et al., 2011). In all cases, the mechanism of action of GH3 proteins was explained by their modulating effect on the concentration of free IAA or other defense-related hormones. In this study, we address the role of conjugated forms of IAA in promoting plant disease development caused by two different pathogens. Our work shows that infection with *Botrytis cinerea* and *Pseudomonas syringae* leads to transcriptional activation of auxin conjugating *GH3.2* and consequently to accumulation of IAA-Asp in *Arabidopsis*. We also show that this conjugated auxin promotes disease development in several plant species. Finally, we demonstrate that IAA-Asp increases pathogen progression in the plant by regulating the transcription of virulence genes.

## RESULTS

### *B. cinerea* and *Pst* DC3000 Induce the Accumulation of the Conjugated Auxin IAA-Asp in *Arabidopsis*

We first investigated whether *Arabidopsis* accumulates conjugated forms of IAA in response to the necrotroph *B. cinerea* and the hemibiotroph *P. syringae* pv *tomato* strain DC3000 (*Pst* DC3000) plant pathogens. We focused on the amino acid–conjugated forms IAA-Ala, IAA-Asp, IAA-Glu, and IAA-Leu, which are the most abundant in plants (Ludwig-Müller, 2011). *Arabidopsis* plants were

sprayed with  $5 \times 10^5$  spores/mL of *B. cinerea* or vacuum infiltrated with  $10^6$  colony-forming units (cfu)/mL of *Pst* DC3000, and samples were harvested at 0, 12, 24, and 48 h after inoculation (HAI) for quantification of IAA–amino acid conjugated forms using HPLC–liquid chromatography–tandem mass spectrometry. Interestingly, of the four conjugated forms tested, only IAA-Asp showed a significant increased accumulation. Compared to mock inoculation, IAA-Asp level was, on average, 10-fold higher in plants inoculated with *B. cinerea* at 48 HAI and 1.8-fold higher in plants inoculated with *Pst* DC3000 at 12 HAI (Table 1).

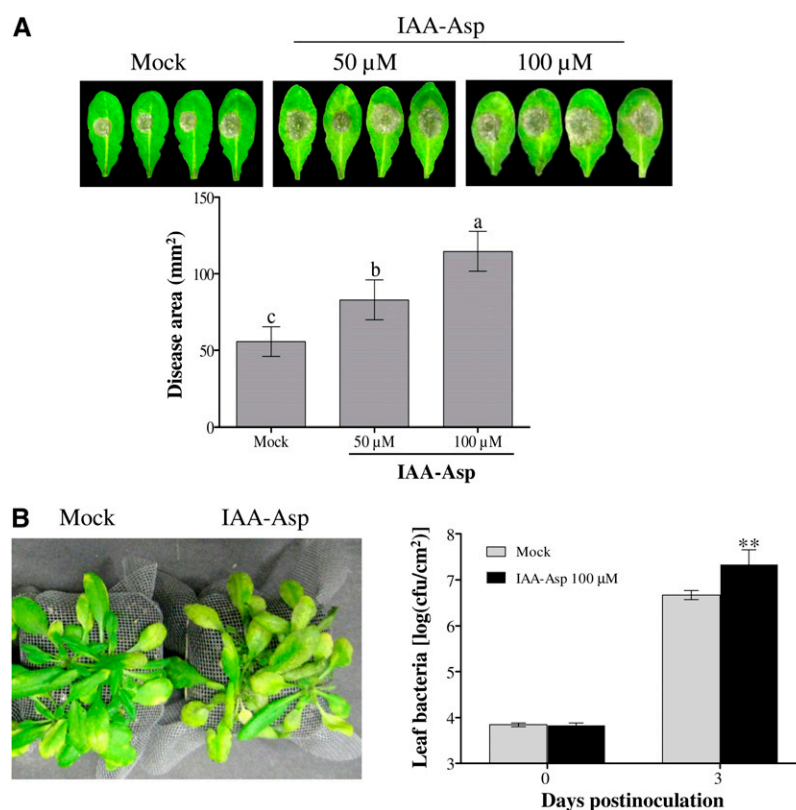
### IAA-Asp Enhances Disease Caused by *B. cinerea* and *Pst* DC3000

To test whether IAA-Asp has an effect on the development of disease, 5-week-old wild-type plants were pretreated with 50 or 100  $\mu$ M of IAA-Asp before infection with *B. cinerea*, and disease development was analyzed 2 d after inoculation (DAI) by measuring lesion areas. Figure 1A shows that both concentrations of IAA-Asp enhanced disease caused by *B. cinerea*, with the increase being most marked with the 100  $\mu$ M pretreatment. Consequently, this concentration was selected for further studies. The effect of IAA-Asp on infection by *Pst* DC3000 was also tested. Plants were vacuum infiltrated with a solution containing 10 mM MgCl<sub>2</sub>, 100  $\mu$ M IAA-Asp, and  $10^6$  cfu/mL of *Pst* DC3000, and leaf discs were harvested for bacterial quantification at 0 and 3 d after infiltration. Plants treated with a solution containing 10 mM MgCl<sub>2</sub>, ethanol used to solubilize the IAA-Asp, and  $10^6$  cfu/mL of *Pst* DC3000 were included as controls. Figure 1B shows that IAA-Asp–treated plants had more symptoms and 8 times more bacteria than control plants. These results therefore indicate that IAA-Asp increases disease symptoms and development caused by the necrotroph *B. cinerea* and the hemibiotroph *Pst* DC3000 in *Arabidopsis*. We then tested whether IAA-Asp enhances disease in other plant species. Leaves of tobacco (*Nicotiana benthamiana*) and tomato (*Solanum lycopersicum* cv MoneyMaker) were syringe infiltrated with a solution containing 10 mM MgCl<sub>2</sub>, 100  $\mu$ M IAA-Asp, and  $10^7$  cfu/mL of *Pst* DC3000, and leaf discs were harvested for bacterial quantification

**Table 1.** Concentration of Amino Acid–Conjugated IAA in Response to Inoculation with *B. cinerea* or *Pst* DC3000

Treatment	IAA-Ala (ng/g DW)	IAA-Asp (ng/g DW)	IAA-Glu (ng/g DW)	IAA-Leu (ng/g DW)
Col-0 0	n.d.	22 $\pm$ 2	n.d.	n.d.
<i>B. cinerea</i>				
Col-0 24 HAI mock	n.d.	17 $\pm$ 6	12 $\pm$ 2	n.d.
Col-0 24 HAI B.c.	n.d.	19 $\pm$ 6	11 $\pm$ 3	n.d.
Col-0 48 HAI mock	n.d.	23 $\pm$ 6	24 $\pm$ 6	n.d.
Col-0 48 HAI B.c.	n.d.	<b>225 <math>\pm</math> 73</b>	21 $\pm$ 5	n.d.
<i>Pst</i> DC3000				
Col-0 12 HAI mock	n.d.	23 $\pm$ 6	24 $\pm$ 4	n.d.
Col-0 12 HAI <i>Pst</i>	n.d.	<b>43 <math>\pm</math> 14</b>	19 $\pm$ 5	n.d.
Col-0 24 HAI mock	n.d.	21 $\pm$ 4	18 $\pm$ 4	n.d.
Col-0 24 HAI <i>Pst</i>	n.d.	26 $\pm$ 4	21 $\pm$ 5	n.d.
Col-0 48 HAI mock	n.d.	n.d.	n.d.	n.d.
Col-0 48 HAI <i>Pst</i>	n.d.	n.d.	n.d.	n.d.

Plants were inoculated with either *B. cinerea* (B.c.) or *Pst* DC3000 (*Pst*) or mock inoculated, and samples were harvested at 0, 24, and 48 HAI (B.c.) or 0, 12, 24, and 48 HAI (*Pst*) for amino acid–conjugated IAA quantification, as described in Methods. Values reported as ng/g of dry weight (DW) are the means of two independent experiments ( $\pm$ SD). n.d., not detected, in which values were below the limit of quantification, with a signal-to-noise ratio of <3. Bold terms correspond to values significantly different between mock treated and infected plants.



**Figure 1.** IAA-Asp Enhances Disease Caused by *B. cinerea* and *Pst* DC3000 in *Arabidopsis*.

**(A)** Col-0 plants were pretreated with two different concentrations of IAA-Asp, and 2 h later, leaves were detached for inoculation with *B. cinerea*. Disease areas were quantified 2 DAI ( $n = 30$ ). Plants treated with ethanol to solubilize the hormone before inoculation with *B. cinerea* were used as mock. Disease areas were analyzed using one-way ANOVA as described in Methods. Within each figure, letters above bars indicate statistical significance; bars not sharing letters represent significant mean differences as assessed by Duncan's multiple range test at  $P < 0.01$ . Experiments were repeated at least three times with similar results.

**(B)** Five-week-old plants were vacuum infiltrated with a solution containing 10 mM  $\text{MgCl}_2$ , 100 mM IAA-Asp, and  $10^6$  cfu/mL of *Pst* DC3000. Left panel, symptoms at 3 DAI. Right panel, leaf discs were harvested for bacterial quantification at 0 and 3 d after vacuum infiltration as described in Methods. Plants treated with a solution containing 10 mM  $\text{MgCl}_2$ , ethanol used to solubilize the hormone, and  $10^6$  cfu/mL of *Pst* DC3000 were used as a mock. Bacteria were recovered from leaf discs and growth determined as cfu/cm<sup>2</sup> of leaf tissue. Error bars represent SD ( $n = 9$ ). Data sets marked with asterisks are significantly different from mock-inoculated leaves as assessed by Turkey's studentized range test (HSD) at  $^{**}P < 0.01$ . Experiments were repeated at least three times with similar results.

at 0 and 3 d after infiltration. Control plants were syringe infiltrated with a solution containing 10 mM  $\text{MgCl}_2$ , ethanol used to solubilize the IAA-Asp, and  $10^7$  cfu/mL of *Pst* DC3000. Supplemental Figure 1 online shows that IAA-Asp promotes *Pst* DC3000 bacterial growth in tomato and *N. benthamiana*. IAA-Asp-treated tomato and *N. benthamiana* plants had around 8 and 10 times more bacteria than control plants, respectively. Thus, the effect of IAA-Asp on plant disease is not restricted to *Arabidopsis*.

### GH3.2 Promotes Disease Caused by *B. cinerea* and *Pst* DC3000 through the Accumulation of IAA-Asp

We then verified whether infection led to induction of the *GH3* genes responsible for the synthesis of IAA-Asp. We measured transcript levels for *GH3.2*, *GH3.3*, *GH3.4*, *GH3.5*, *GH3.6*, and *GH3.17* as all these genes have been shown to encode proteins that conjugate Asp to IAA in vitro (Staswick et al., 2005). Plants were sprayed with

*B. cinerea* spores or vacuum infiltrated with *Pst* DC3000 as described above, and samples were harvested for RNA extraction at 0, 12, and 24 HAI. Mock-inoculated plants were included as controls. As shown in Figure 2 and Supplemental Figure 2 online, only *GH3.2* and *GH3.3* transcript levels were significantly increased by both pathogens compared to mock infection, with *GH3.2* showing the highest increase (66-fold) after infection by *B. cinerea* at 24 HAI. We then took advantage of the availability of *Arabidopsis* mutants for the *GH3.2*, *GH3.4*, *GH3.5*, *GH3.6*, and *GH3.17* genes (see Supplemental Figure 3 online) to further characterize the role of *GH3* proteins in the resistance against *B. cinerea* and *Pst* DC3000. Figures 3A and 3B show that, compared to wild-type plants, the *gh3.2* mutant, in both the *Landsberg erecta* (Ler) and Columbia-0 (Col-0) background, is highly resistant to *B. cinerea*. Of the other *gh3* mutants tested, only *gh3.4* displayed a slight but significant increased resistance (Figure 3B). Nevertheless, the infection was more severe in the *gh3.4* plants than in the *gh3.2* mutant. The *gh3.2*

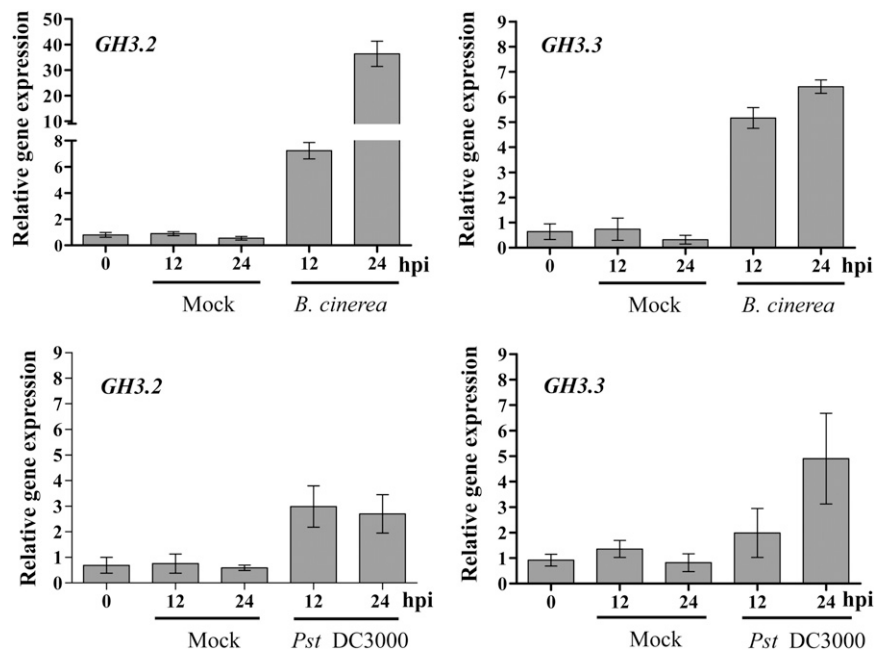
mutant was also significantly more resistant than the wild type to infection by *Pst* DC3000 (Figures 3C and 3D). To verify whether the increased resistance displayed by *gh3.2* plants was linked to a diminution in the amount of IAA-Asp, we then measured the accumulation of this metabolite in *Pst* DC3000- and *B. cinerea*-inoculated plants 12 and 48 HAI, respectively. Figure 4 shows that the levels of IAA-Asp were significantly reduced in the *gh3.2* mutant compared to wild-type plants in response to both pathogens, suggesting that the induction of IAA-Asp occurs mainly through GH3.2. Altogether, our results suggest that IAA-Asp accumulates through the action of the GH3.2 protein and promotes disease caused by both pathogens. To confirm this, we tested whether IAA-Asp could rescue the reduced disease susceptible phenotype of the *gh3.2* mutant. Plants were pre-treated with 100  $\mu$ M IAA-Asp and, 2 h later, leaves were detached and inoculated with *B. cinerea* as described in Methods. IAA and the synthetic auxin 2,4-D were also included in these experiments as controls. Figure 5 indicates that IAA-Asp and free IAA, but not 2,4-D, enhance disease caused by *B. cinerea* in wild-type plants. However, only IAA-Asp could rescue the *gh3.2* phenotype (Figures 5A and 5B), suggesting that the higher infection observed in wild-type plants after treatment with IAA is mainly due to its conversion to IAA-Asp. Similar results were obtained upon infection with *Pst* DC3000 (Figure 5C). Again, IAA and IAA-Asp enhanced disease caused by the pathogen, but only IAA-Asp was able to rescue the *gh3.2* phenotype (Figure 5C). 2,4-D was not included in this experiment as this synthetic

auxin is already known to induce symptoms upon infection by this pathogen but not bacterial growth (Navarro et al., 2006).

### The SA, JA, and Camalexin Pathways Do Not Mediate the *gh3.2*-Reduced, Disease-Susceptible Phenotype

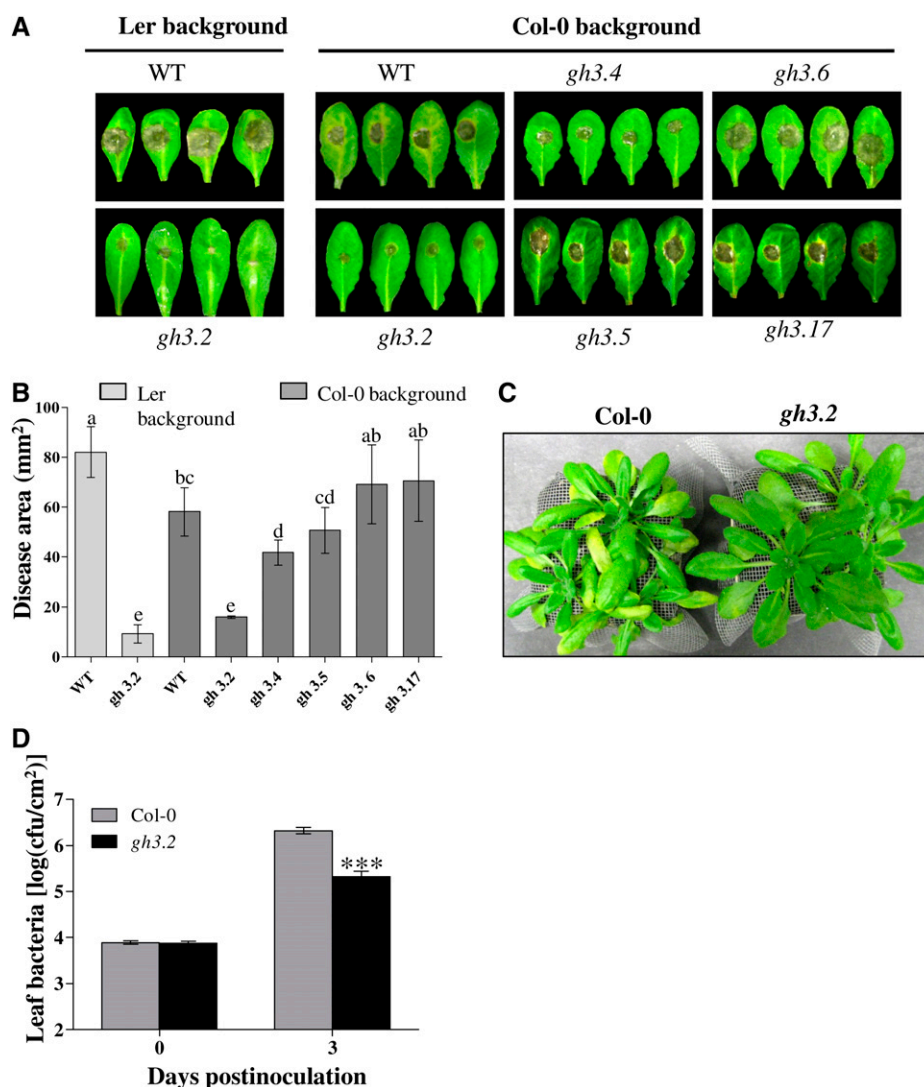
To test the possibility that the disease resistance observed in *gh3.2* is mediated by SA (pathway required for resistance against *Pst* DC3000) or JA (pathway required for resistance against *B. cinerea*), we quantified by quantitative PCR (qPCR) the expression levels of *PR1*, *PR2*, and *PR5* (markers of the SA pathway) in plants inoculated with *Pst* DC3000 and of *PDF1*, *Basic Chitinase (CHB)*, and *Thionine 2.1 (Thio2.1)* (markers of the JA pathway) in *B. cinerea*-inoculated plants. Supplemental Figure 4 online shows that, after infection with the respective pathogens, the pattern of *PR1*, *PR2*, and *PR5* expression is similar in both wild-type and *gh3.2* plants. However, levels of *PDF1*, *CHB*, and *Thio2.1* transcripts are significantly lower in the mutant background. Therefore, although *GH3.2* might positively regulate the JA pathway, this putative role cannot explain the higher resistance of the *gh3.2* mutant. These results suggest that the SA and JA pathways are not involved in the *gh3.2*-reduced, disease-susceptible phenotype.

The phytoalexin camalexin is one of the major components of *Arabidopsis* defense against *B. cinerea* (Ferrari et al., 2003; Kliebenstein et al., 2005). To test if the reduced disease-susceptible phenotype of the *gh3.2* mutant is related to a defect in the accumulation of camalexin, we determined camalexin levels in inoculated



**Figure 2.** *B. cinerea* and *Pst* DC3000 Induce the Expression of *GH3.2* and *GH3.3*.

Col-0 plants were either sprayed with spores of *B. cinerea* or vacuum infiltrated with *Pst* DC3000 as described in Methods. Samples were harvested at 0, 12, and 24 HAI for qPCR analysis. Water and 10 mM  $\text{MgCl}_2$  were used as mock inoculation for *B. cinerea* and *Pst* DC3000, respectively. Primers used to quantify *GH3.2* and *GH3.3* transcripts are described in Supplemental Table 1 online. Error bars represent SD from three biological replicates. Each biological replicate contains a pool of five plants. Data sets marked with asterisks are significantly different from mock-inoculated plants at the same time point as assessed by the Tukey's studentized range test (HSD, \*\* $P < 0.01$ ). Experiments were repeated at least three times with similar results.



**Figure 3.** GH3.2 Promotes Disease Caused by *B. cinerea* and *Pst* DC3000.

(A) and (B) Detached leaves from *gh3* mutants and their corresponding wild type (WT; Col-0 and Landsberg *erecta*) were inoculated as described in Methods. Leaf photographs were taken at 2 DAI. Disease areas were also measured in each leaf (B). Error bars represent SD ( $n = 20$ ). Disease areas were analyzed using one-way ANOVA. Within each figure, letters above bars indicate statistical significance; bars not sharing letters represent significant mean differences as assessed by Duncan's multiple range test at  $P < 0.01$ .

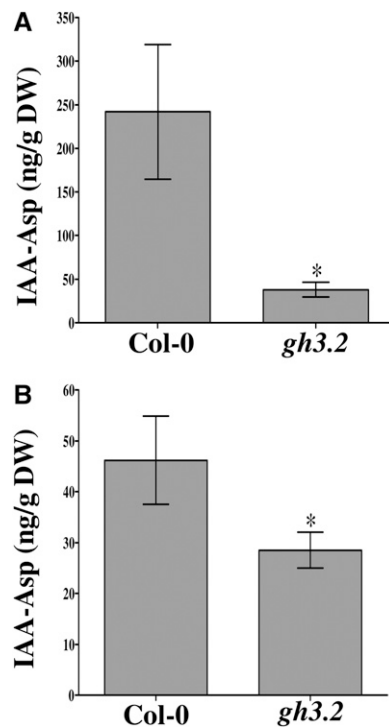
(C) and (D) Wild-type and *gh3.2* plants were vacuum infiltrated with 10 mM MgCl<sub>2</sub> containing 10<sup>6</sup> cfu/mL of *Pst* DC3000. Symptoms at 3 DAI (C); bacterial growth was analyzed as described in Methods (D). Data sets marked with asterisks are significantly different from inoculated wild-type plants as assessed by Student's *t* test: \*\*\* $P < 0.001$ . Experiments were repeated at least three times with similar results.

plants. Camalexin levels were similar in both genotypes at 0, 24, and 48 HAI (see Supplemental Figure 4 online). These results suggest that camalexin is not involved in the *gh3.2*-reduced, disease-susceptible phenotype.

#### PTI Does Not Mediate the *gh3.2*-Reduced, Disease-Susceptible Phenotype

We next investigated whether GH3.2 suppresses PTI, which could explain the reduced disease-susceptible phenotype ob-

served in the *gh3.2* mutant. PAMPs, including flagellin (Flg22), are able to inhibit root elongation and seedling growth; consequently, these two responses are usually used as markers of PTI (Gómez-Gómez et al., 1999; Zeng and He, 2010). Wild-type and *gh3.2* seeds were grown in Murashige and Skoog (MS) medium supplemented with 10  $\mu$ M Flg22. Root lengths were measured and seedlings were weighed 10 d after treatment as described in Methods. Figures 6A to 6C show that the inhibitory effect of Flg22 on root lengths and seedling growth was similar for both genotypes. We then investigated whether GH3.2 suppressed the expression of the Flg22-induced



**Figure 4.** IAA-Asp Accumulation Is Significantly Compromised in *gh3.2* Plants in Response to Infection by Both Pathogens.

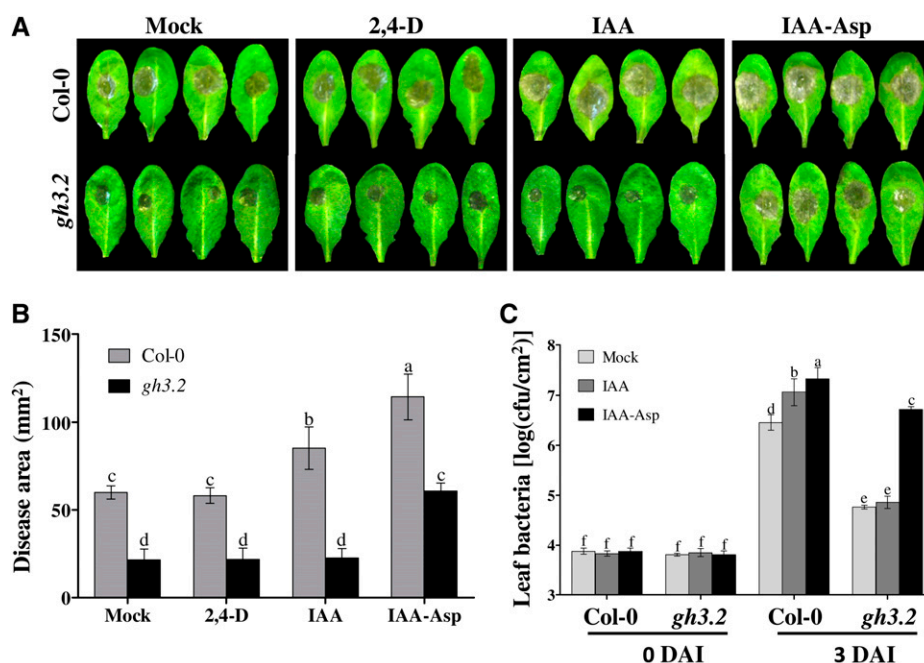
(A) and (B) Col-0 and *gh3.2* plants were inoculated with either *B. cinerea* or *Pst DC3000*, and samples were harvested 48 HAI (A; *B. cinerea*) and 12 HAI (B; *Pst DC3000*). IAA-Asp was quantified as described in Methods. Error bars represent SD ( $n = 3$  in [A] and  $n = 5$  in [B]). Data set marked with an asterisk is significantly different from inoculated wild-type plants as assessed by Tukey's studentized range test (HSD): \* $P < 0.05$ . DW, dry weight.

markers WRKY22 and FRK1 (for Flagellin-Responsive Kinase1) (Asai et al., 2002). Wild-type and *gh3.2* mutant seeds were grown in MS medium and then treated with Flg22 as described in Methods. Seedlings were harvested for RNA extraction at 0, 30, and 60 min after treatment. qPCR analysis showed that the levels of WRKY22 and FRK1 transcripts were slightly reduced in *gh3.2*-treated seedlings compared to wild-type-treated seedlings (Figure 6D). These results suggest that GH3.2 might positively regulate the expression of some Flg22-induced markers. We then investigated whether Flg22 regulates the expression level of GH3.2. Col-0 seeds were grown in MS medium before treatment with 10  $\mu$ M Flg22. Samples were harvested at 0, 30, and 60 min, and RNA was extracted. Mock-treated seedlings were included as controls. qPCR indicated that Flg22 induces the expression of GH3.2 at 60 min after treatment (Figure 6E). Thus, globally, our results indicate that the PTI pathway does not regulate the *gh3.2*-reduced, disease-susceptible phenotype.

#### IAA-Asp-Induced Virulence of *B. cinerea* and *Pst DC3000* Occurs in Planta

Our results indicate that IAA-Asp favors plant infection by pathogens. One possible explanation is that IAA-Asp acts as a direct

inducer of pathogen virulence or growth. To test that, we first determined whether the addition of IAA-Asp to the culture medium activated the growth of *Pst DC3000* or the germination of spores and the growth of germ tubes of *B. cinerea*. For *Pst DC3000*, a suspension of  $10^6$  cfu/mL bacteria was incubated in minimal medium supplemented with 100  $\mu$ M IAA-Asp, and samples were taken at different time points for bacterial quantification. Mock-treated bacteria were used as control. Supplemental Figure 5A online shows that IAA-Asp did not enhance bacterial growth at the concentration tested. We then analyzed by qPCR the expression of the effectors *AvrPto*, *HopU1*, and *Hop-AO1*, which are known to be important for *P. syringae* infection (Underwood et al., 2007; Cunnac et al., 2009; Hann et al., 2010). Expression of these genes was similar whether IAA-Asp was present or not in the medium (see Supplemental Figure 5B online). Similar experiments were done with *B. cinerea* using potato dextrose broth medium to test whether IAA-Asp accelerates the germination of *B. cinerea* spores and the growth of their germ tubes. The results indicate that IAA-Asp had no effect on these growth parameters (see Supplemental Figures 5C and 5D online). We then analyzed by qPCR the effect of IAA-Asp on the expression of three genes involved in virulence of *B. cinerea*, the transporter *BcatrB*, the xylanase *Xyn11A*, and the tetraspanin *Bcpls1* (Brito et al., 2006; Veneault-Fourrey et al., 2006; Choquer et al., 2007; Stefanato et al., 2009). No change in expression was observed for any of these genes (see Supplemental Figure 5E online). These results suggest that IAA-Asp does not activate growth or virulence of *Pst DC3000* and *B. cinerea* in vitro. However, as the expression of bacterial pathogen virulence effectors often requires direct contact of the bacteria with the invaded host (Pizarro-Cerdá and Cossart, 2006; Bhavsar et al., 2007), we decided to measure the expression level of the effector and virulence genes described above during infection in wild-type and *gh3.2* plants. Plants were inoculated with *Pst DC3000* as described in Methods, and expression of bacterial virulence genes was quantified in planta by qPCR. The inoculation time point showing the highest difference in IAA-Asp accumulation between Col-0 and *gh3.2* plants (12 HAI) was chosen for analysis. Results indicate that, while the expression of *HopU1* was similar in both genotypes, the expression of *AvrPto* and *HopAO1* was significantly reduced in *gh3.2* plants (Figure 7). Similar experiments conducted with *B. cinerea* at 48 HAI indicated that the *BcatrB* gene expression, but not that of the two other genes tested, was also repressed in the *gh3.2* plants (Figure 7). These results suggest that GH3.2 is involved in activating the expression of some virulence genes in both pathogens and that this action is mediated by IAA-Asp. This was verified by analyzing the expression of the same virulence genes in wild-type and *gh3.2* plants pretreated with IAA-Asp before infection. Plants were infiltrated with IAA-Asp before inoculation with each of the pathogens and samples were taken 24 HAI to verify the expression of the virulence genes. Results in Figure 8 show that IAA-Asp induced the expression of *AvrPto* and *HopAO1* in inoculated wild-type and *gh3.2* plants. Although the level of *HopU1* transcripts was also increased at 24 HAI in pretreated wild-type and *gh3.2* plants, this level was not different from that observed in both genotypes at 0 HAI (Figure 8). In *B. cinerea*, the *BcatrB* expression was reduced at 24 HAI in mock-treated *gh3.2* plants but was highly induced in IAA-Asp-pretreated



**Figure 5.** GH3.2 Contributes to Disease Caused by *B. cinerea* and *Pst* DC3000 through IAA-Asp.

**(A)** and **(B)** Col-0 and *gh3.2* plants were vacuum infiltrated with 100  $\mu$ M of 2,4-D, IAA, or IAA-Asp and 2 h later, leaves were detached and inoculated with spores of *B. cinerea*. A solution of ethanol diluted in water was used as a mock as described in Methods. Photographs of leaves were taken at 2 DAI **(A)**, and disease areas were measured in each leaf **(B)**. Error bars represent SD ( $n = 20$ ). Disease areas were analyzed using two-way ANOVA. Within each figure, letters above bars indicate statistical significance; bars not sharing letters represent significant mean differences as assessed by Duncan's multiple range test at  $P < 0.01$ .

**(C)** Plants were vacuum infiltrated with 10 mM  $MgCl_2$  containing either 100  $\mu$ M of IAA or IAA-Asp and  $10^6$  cfu/mL of *Pst* DC3000, and bacterial growth was analyzed 0 and 3 DAI as described in Methods. Mock plants were also included as described in Methods. Leaf bacteria were analyzed using two-way ANOVA. Within each figure, letters above bars indicate statistical significance; bars not sharing letters represent significant mean differences as assessed by Duncan's multiple range test at  $P < 0.01$ . Experiments were repeated at least three times with similar results.

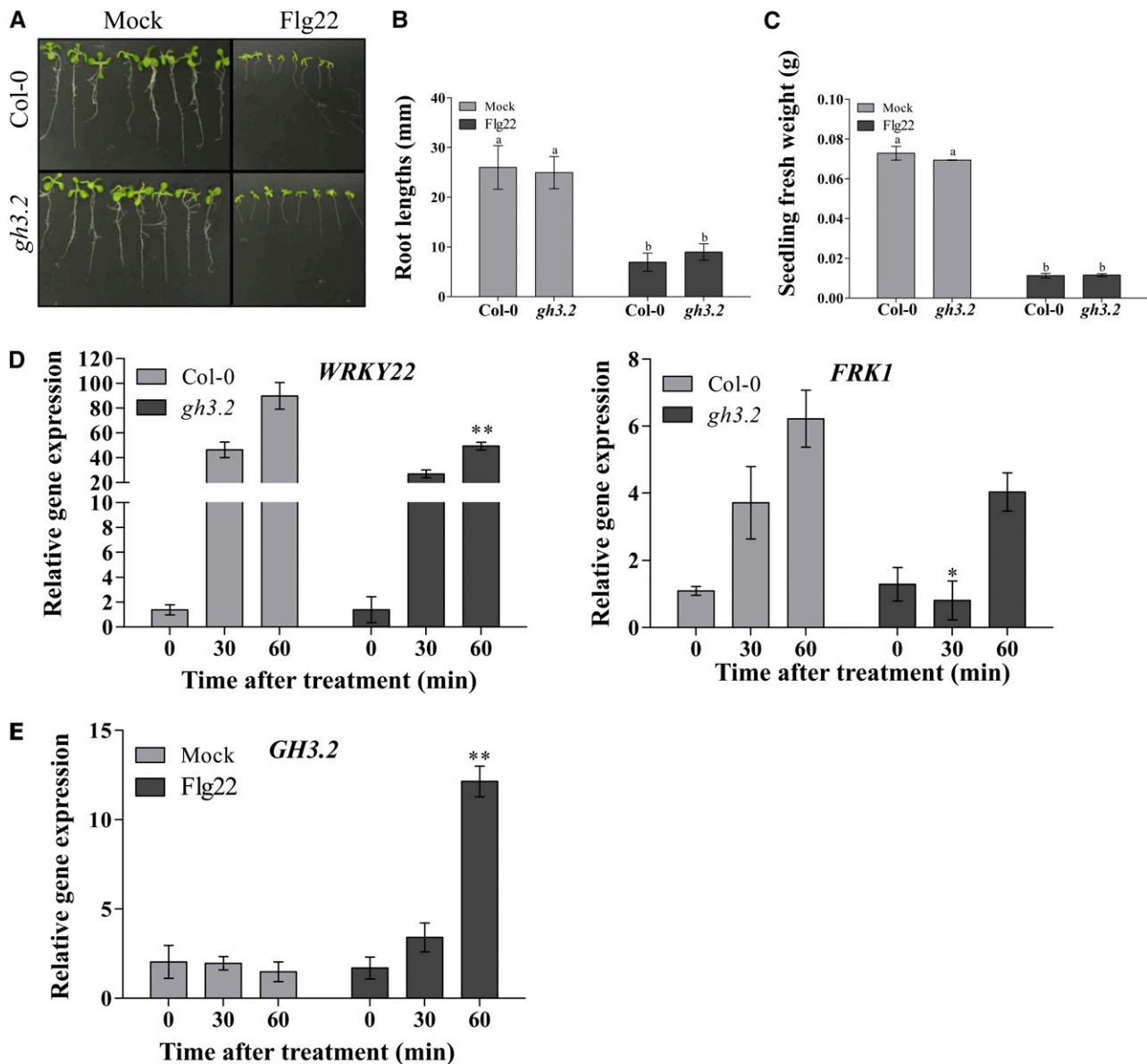
plants (Figure 8). The expression level of *Xyn11A* was comparable in mock-treated wild-type and *gh3.2* plants at 0 and 24 HAI but increased at 24 HAI in both IAA-Asp-pretreated genotypes (Figure 8). However, IAA-Asp did not affect the expression level of *Bcpls1* in either genotype at 0 and 24 HAI. These results strongly suggest that IAA-Asp promotes disease by activating pathogen virulence and that this requires direct contact between the plant and the pathogen or a plant component.

## DISCUSSION

Our results indicate that GH3.2 and IAA-Asp play an important role in promoting disease. While previous studies reported a function for GH3 genes in disease resistance (Ludwig-Müller, 2011), a link between disease susceptibility and the accumulation of IAA-Asp was not reported. For example, a mutant of GH3.5, which uses both IAA and SA as substrates, was shown to confer resistance to *P. syringae* through defense responses involving the expression of PR1 (Park et al., 2007; Zhang et al., 2007), suggesting that its function involves alteration of SA homeostasis (Zhang et al., 2007). The same study revealed that an activation-tagged dwarf mutant of GH3.6 leads to increased

sensitivity to *P. syringae* in *Arabidopsis*, but the level of IAA-Asp after infection was not determined in this work (Zhang et al., 2007). GH3-11 and GH3-12 are also involved in plant defense, but they do not conjugate IAA (Staswick et al., 2005; Jagadeeswaran et al., 2007; Nobuta et al., 2007). In rice, Os-GH3-8 contributes to plant defense via reduction of the free IAA concentration, which positively regulates the expression of expansin. As overexpression of expansin in rice has been shown to increase susceptibility to *Xanthomonas oryzae* pv *oryzae*, this provides a mechanism by which Os-GH3-8 confers disease resistance (Ding et al., 2008). Also in rice, a homolog of GH3.2 has recently been proposed to positively regulate disease resistance, presumably by suppressing the action of IAA, thus preventing the production of expansins (Fu et al., 2011). However, this conclusion was based on Os-GH3.2 constitutive overexpressor lines that showed little increase in IAA-Asp levels compared to wild-type plants. Moreover, the concentration of conjugated IAA did not change during infection with two of the three pathogens tested, while only a small accumulation of IAA-Asp was detected with the third pathogen. This suggests that conjugation of IAA with Asp is not the main function of this protein during rice-pathogen interaction. Furthermore, as Os-GH3-2 RNA interference lines with up to 79% reduction in Os-GH3-2





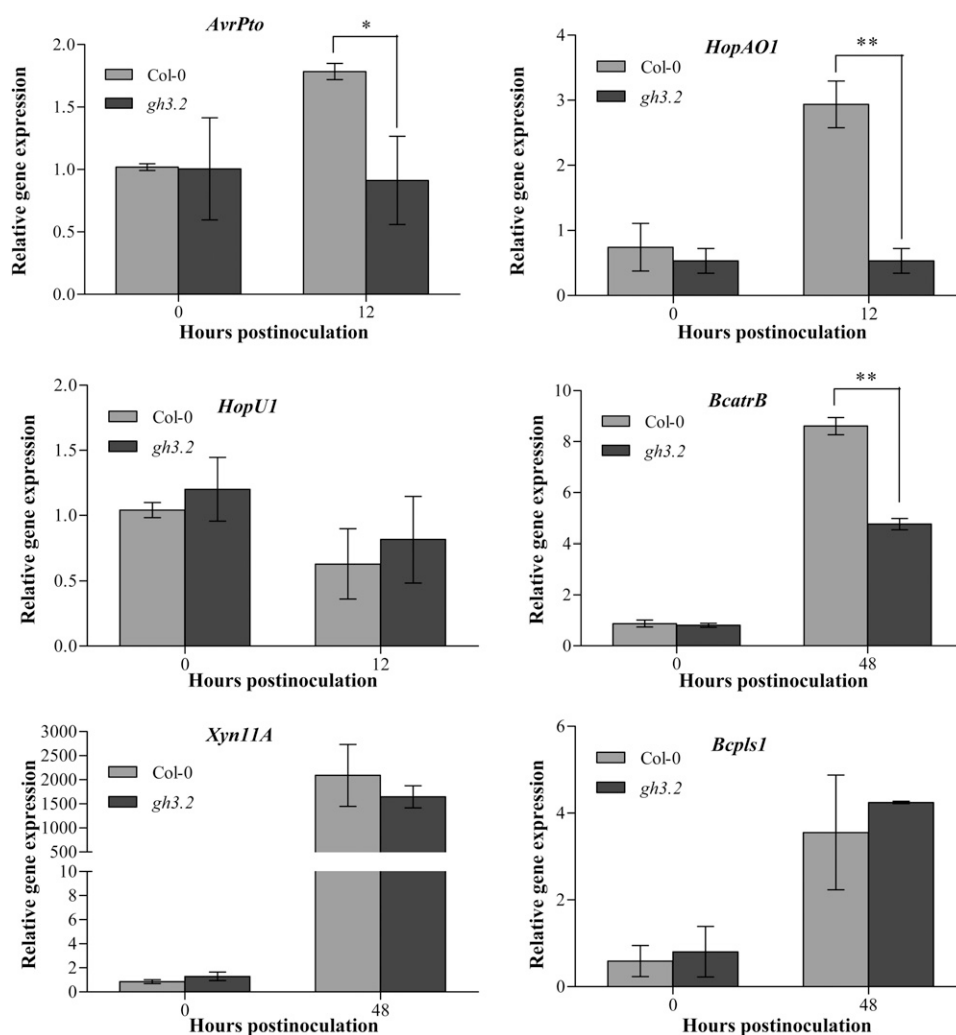
**Figure 6.** PTI Is Not Involved in the Reduced Disease-Susceptible Phenotype Observed in *gh3.2* Plants.

(A) to (C) Col-0 and *gh3.2* seeds were grown in MS medium supplemented with 10  $\mu$ M Flg22. Root length (A) and (B) was measured and seedlings were weighed 10 d after germination (C). Error bars represent SD from two independent experiments ( $n = 20$ ). Within each figure, letters above bars indicate statistical significance; bars not sharing letters represent significant mean differences as assessed by Duncan's multiple range test at  $P < 0.01$ . (D) Col-0 and *gh3.2* seeds were grown in MS medium before treatment with 10  $\mu$ M Flg22. Samples were harvested at 0, 30, and 60 min, and RNA was extracted as described in Methods. qPCR analysis was carried out to determine the expression level of the Flg22-induced markers, *WRKY22* and *FRK1*. (E) Col-0 seeds were grown in MS medium before treatment with 10  $\mu$ M Flg22. Samples were harvested at 0, 30, and 60 min, and RNA was extracted as explained in Methods. Mock-treated seedlings were included in this experiment. qPCR analysis was carried out to determine the expression level of *GH3.2*.

(D) and (E) Relative gene expressions were analyzed using one-way ANOVA. Error bars represent SD from three biological replicates. Each biological replicate contained a pool of 10 seedlings. Data sets marked with asterisks are significantly different from wild-type plants (D) or mock-treated plants (E) at the same time point as assessed by Tukey's studentized range test (HSD): \* $P < 0.05$  or \*\* $P < 0.01$ . The experiments were repeated twice with similar results.

[See online article for color version of this figure.]





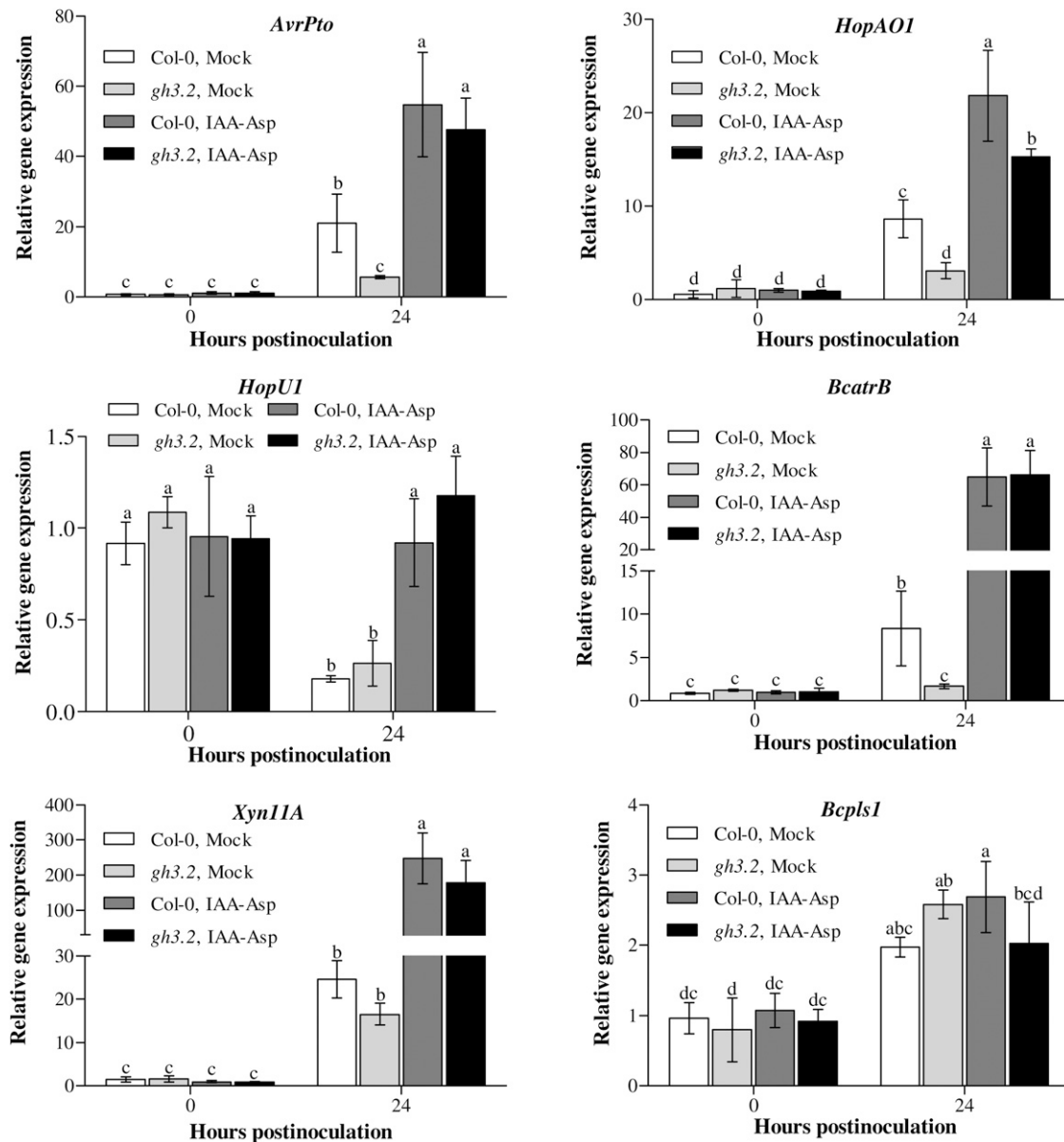
**Figure 7.** GH3.2 Contributes to the Activation of Some Virulence Factors of *Pst* DC3000 and *B. cinerea*.

*Col-0* and *gh3.2* plants were inoculated with either *B. cinerea* (*BcatrB*, *Xyn11A*, and *Bcpls1*) or *Pst* DC3000 (*AvrPto*, *HopAO1*, and *HopU1*). Samples were harvested at 0 and 12 HAI for *Pst* DC3000 and at 0 and 48 HAI for *B. cinerea*, and RNA was extracted as described in Methods. qPCR analysis was carried out to determine the expression level of the *Pst* DC3000 and *B. cinerea* virulence factors described in Supplemental Figure 4 online. Relative gene expression was analyzed using two-way ANOVA. Error bars represent SD of three biological replicates. Each biological replicate contains a pool of five plants. Data sets marked with asterisks are significantly different from wild-type plants at the same time point as assessed by the t test (LSD) and Tukey's studentized range test (HSD): \* $P < 0.05$  or \*\* $P < 0.01$ . The experiment was repeated at least three times with similar results.

transcript level reacted similarly to wild-type plants to infection (Fu et al., 2011), it cannot be excluded that the resistance phenotype observed in the overexpressor lines is due to a pleiotropic effect of *Os-GH3.2* constitutive overexpression.

Auxin signaling has long been suspected to benefit pathogens. Indeed, several studies have demonstrated the importance of the different components of the auxin signaling pathway in resistance against biotrophs and necrotrophs (El Oirdi and Bouarab, 2007; Yang et al., 2007; Spoel and Dong, 2008; Cuzick et al., 2009; Truman et al., 2010; Kidd et al., 2011). Nevertheless, a direct, major role for IAA in plant susceptibility has not yet been demonstrated. For example, while pretreatment of *Arabidopsis* with the synthetic auxins 2,4-D and 1-naphthaleneacetic acid (NAA) was shown to

increase disease symptoms to *Pst* DC3000, bacterial growth curves were similar to those from nontreated control plants (Navarro et al., 2006; Chen et al., 2007). Actually, only the growth of *P. syringae* pv *maculicola* was shown to moderately increase following pretreatment with NAA (Wang et al., 2007). Thus, while the synthetic auxin 2,4-D does not appear to favor bacterial growth, our work shows a direct effect for the natural auxin IAA and one of its derivatives in promoting bacterial growth in *Arabidopsis*. This difference between natural and synthetic auxins may be due to the substrate specificity of GH3 proteins. Indeed, while several members of the GH3 family in *Arabidopsis* are able to conjugate amino acids to IAA and other natural (indole-3-pyruvic acid and indole-3-butyric acid) and synthetic (NAA) auxins, none of these proteins can conjugate 2,4-D in



**Figure 8.** IAA-Asp Activates the Expression of Some Virulence Factors of *Pst* DC3000 and *B. cinerea* in Planta.

*Col-0* and *gh3.2* plants were pretreated with IAA-Asp before they were inoculated with *B. cinerea* (*BcatrB*, *Xyn11A*, and *Bcpls1*) as described in Methods. Pretreatment with a solution of ethanol was used as a mock. Similar experiments were carried out with *Pst* DC3000 (*AvrPto*, *HopAO1*, and *HopU1*), and mock inoculation was included as described in Methods. Samples were harvested at 0 and 24 HAI for RNA extraction as described in Methods. qPCR analysis was carried out to determine the expression level of the *Pst* DC3000 and *B. cinerea* virulence factors described in the Figure 7 and Supplemental Figure 5 online. Error bars represent SD from three biological replicates. Each biological replicate contained a pool of five plants. Experiments were repeated at least three times with similar results. Expression levels were analyzed using two-way ANOVA. Within each figure, letters above bars indicate statistical significance; bars not sharing letters represent significant mean differences as assessed by Duncan's multiple range test at  $P < 0.05$ . Experiments were repeated at least three times with similar results.

vitro (Staswick et al., 2005). This raises the possibility that it is the conjugated form(s) and not the free auxin that has a role in plant susceptibility to pathogens. This hypothesis is now supported by our data showing a direct effect of IAA-Asp on disease susceptibility and that the auxin-conjugating enzyme GH3.2 is important to mediate this effect. While other GH3 proteins might also contribute

to disease susceptibility during infection, their contribution appears to be minor compared to that of GH3.2.

We tried to determine how IAA-Asp might mediate disease susceptibility by first looking at modulation of well-characterized defense pathways in the *gh3.2* plants upon inoculation. None of the markers used in this study indicated that these pathways are

involved in the reduced disease susceptible phenotype of *gh3.2*. Recently, the brassinosteroid pathway, which is involved in *Brassicaceae* plant development, has been shown to compromise PTI (Albrecht et al., 2011; Belkadir et al., 2011). Our results indicate that PTI is not involved in the reduced disease susceptibility of *gh3.2* plants. We cannot exclude, however, that other plant pathways might be involved in the reduced disease susceptible phenotype of *gh3.2*.

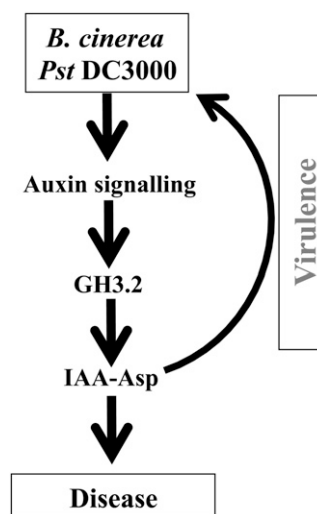
We then tested whether IAA-Asp could exert its action directly on the pathogen. While no effect could be observed on pathogens grown in culture media, IAA-Asp clearly contributed to pathogen growth and virulence gene activation when the measure was made on inoculated plants. The *BcatrB* gene of *B. cinerea* encodes an ABC transporter that increases tolerance to the phytoalexin camalexin and thus promotes virulence. This gene is also known to be induced by camalexin (Stefanato et al., 2009). Interestingly, our results show that *BcatrB* expression is more highly induced in Col-0 than in *gh3.2* plants 48 HAI (Figure 7) but that the camalexin contents remain unchanged under the same conditions (see Supplemental Figure 4 online). These observations suggest that the reduced susceptibility to *B. cinerea* observed in *gh3.2* plants and the induction of *BcatrB* mediated by GH3.2 and IAA-Asp are not related to a modification of the camalexin biosynthesis pathway.

A similar observation was made in *Pst* DC3000 for the virulence genes *AvrPto* and *HopAO1*. These genes encode effectors secreted through the type III secretion system (TTSS) and contribute to pathogen virulence through suppression of host basal defense responses (Dodds and Rathjen, 2010). Our results therefore suggest that the effect of IAA-Asp on disease susceptibility might be exerted on the pathogen, by contributing to the regulation of the transcription of virulence genes. We noted, however, that not all virulence genes tested are induced by pretreatment with IAA-Asp or repressed in *gh3.2* plants. Also, different patterns of transcriptional regulation by IAA-Asp are observed depending on the virulence gene. Therefore, the mechanism of action of IAA-Asp appears to be complex and likely involves interaction with other induction pathways. At this stage, we cannot rule out that GH3.2 might regulate other virulence factors or host responses that contribute to the reduced disease susceptible phenotype observed in *gh3.2* plants.

Recently, Stuttmann et al. (2011) described two different mutants of *Arabidopsis* with increased resistance to the obligate biotrophic pathogen *Hyaloperonospora arabidopsidis*. The authors showed that this resistant phenotype results from an over accumulation of the Asp-derived amino acid Thr and does not appear to be linked to plant immunity (Stuttmann et al., 2011). They also showed that exogenous treatment of plants with Thr enhanced resistance to *H. arabidopsidis*. This raises the possibility that GH3.2, by conjugating Asp to IAA, compromises the accumulation of Thr by reducing the free pool of Asp. One could then envision that the reduced disease susceptible phenotype of the *gh3.2* mutant is due to the combined effect of Thr increase and IAA-Asp diminution. It will be interesting to test this hypothesis in the future. It is unlikely, however, that the increased resistance of the *gh3.2* mutant could be explained only by an increase in the Thr pool as pretreatment with IAA-Asp alone has a direct effect on virulence.

Our results show that the virulence induced by IAA-Asp occurs during plant infection. This induction might require a direct contact between the pathogen and the host or a component of the host. Direct contact is reminiscent of the increased production of bacterial effector proteins translocated by TTSS that occurs when mammalian bacterial pathogens make contact with their host cells (Ménard et al., 1994; Rosqvist et al., 1994; Watarai et al., 1995; Zierler and Galán, 1995; Pizarro-Cerdá and Cossart, 2006). In plants, a well-documented case of contact-dependent expression of bacterial effectors is that of the interaction between *Ralstonia solanacearum* and plant cells from various species (Aldon et al., 2000). In this system, expression of the regulatory *hrpB* gene, which regulates the expression of TTSS biosynthesis genes and effector substrates (Occhialini et al., 2005), is highly induced by a yet unidentified signal present in plant cell walls. Induction of *hrpB* is regulated by PrhA, which appears to act as a receptor for a nondiffusible plant-derived signal (Marenda et al., 1998; Aldon et al., 2000). It is tempting to speculate that the increased pathogenicity linked to the presence of IAA-Asp in *Arabidopsis* could result from its direct interaction with a pathogen-encoded receptor. However, the intracellular localization of IAA-Asp is still unclear and there is no evidence that it could be sequestered in the cell wall.

To date, no biological activity has been ascribed to IAA-Asp, although two studies have hinted toward a role in temperature sensitivity and grape ripening (Oetiker and Aeschbacher, 1997; Böttcher et al., 2010). Our results now show that IAA-Asp is biologically active and promotes disease by two pathogens and in at least three plant species. Thus, these pathogens use a novel strategy to hijack auxin metabolism through the expression of *GH3.2* and the production of IAA-Asp to promote colonization. This is reminiscent of the strategies adopted by other microorganisms,



**Figure 9.** IAA-Asp Promotes Disease in *Arabidopsis* by Activating Pathogen Virulence.

Model showing how *Pst* DC3000 and *B. cinerea* induce the accumulation of IAA-Asp through potentiation of *GH3.2* gene expression, which favors the development of disease by activation of certain virulence factors.

such as *Agrobacteria* and *Rhizobia*, which use small molecules produced by the host to induce virulence or symbiosis (Stachel et al., 1985; Peters et al., 1986). Although we can only speculate on the mechanism by which IAA-Asp activates gene expression in the two pathogens, it is important to note that a link between Trp metabolites, such as IAA, and bacterial pathogenicity has been made before. In *Pseudomonas aeruginosa*, a Gram-negative pathogen that causes acute and chronic infections in humans, IAA, NAA, and 3-hydroxykynurenine were shown to inhibit TTSS expression, thus potentially affecting the virulence of this pathogen (Shen et al., 2008). In *R. solanacearum*, another Trp oxidative metabolite, 3-hydroxy-oxindole, was shown to be synthesized through the concerted action of a six-gene-containing operon under the control of HrpB, itself activated by PhrA and plant cell contact (Delaspre et al., 2007). This metabolite, referred to as HDF (for HrpB-Dependent Factor), was also shown to induce the *Vibrio fischeri* LuxR transcriptional regulator involved in quorum sensing. Although the function of HDF remains obscure, the fact that its synthesis is coordinated with that of effector proteins and that it may affect quorum sensing suggests that it contributes to the virulence of this plant pathogen (Delaspre et al., 2007). Intriguingly, one study has reported that catabolism of IAA-Asp goes through intermediates with structures similar to HDF, such as 3-acetyl-oxindole (Tuominen et al., 1994), raising the possibility that the effect of IAA-Asp on pathogen virulence could be indirect, through one of its metabolites. This could also explain why IAA-Asp alone in the culture media has no effect on the pathogen.

In this context, detection of IAA-Asp (or one of its metabolite), and not free IAA, could have been acquired during evolution as a means to reinforce virulence, and the induction of *GH3.2* could now be carried out by the pathogen, as observed in our study. We cannot rule out the possibility that, originally, the pathogen used IAA to induce its virulence by manipulating other defense-related hormone pathways (see below). The plant would have then induced GH3 proteins after infection to reduce free IAA levels, with the pathogen next evolving to sense and use IAA-Asp to its own benefit.

What is then the role of auxin signaling in our model (Figure 9)? We know that *GH3.2* is induced by auxin (Takase et al., 2004). Therefore, auxin signaling might activate conjugation through transcriptional activation of *GH3.2*. In this context, the repression of auxin signaling by SA will decrease IAA conjugation and, therefore, pathogen virulence (Wang et al., 2007). On the other hand, it has been proposed that auxin signaling and transport are essential to establish systemic acquired resistance (Truman et al., 2010). Therefore, it is possible that specific auxin signaling compounds are involved either in the regulation of the cross talk between IAA and SA, the activation of IAA conjugation, or both.

Several studies have been done in the last 10 years on strategies used by pathogens to overcome plant immune pathways and establish their disease (Bouarab et al., 2002; Hauck et al., 2003; Anderson et al., 2006; Janjusevic et al., 2006; Melotto et al., 2006; Shan et al., 2008; Gimenez-Ibanez et al., 2009; Ntoukakis et al., 2009; El Oirdi et al., 2011). Our results describe a different strategy used by fungal and bacterial pathogens to manipulate auxin metabolism to produce a conjugated auxin and perceive it as a signal to activate its virulence (Figure 9). Our data present a conceptual stride forward in understanding the role of auxin signaling in plant disease. Exciting future challenges will be to examine how

IAA-Asp is perceived by pathogens in planta and if this molecule regulates other virulence factors.

## METHODS

### Plant Material and Growth Conditions

Seeds were grown on soil in a growth chamber (DiaMed Laboratory Supplies) at 23°C with 70% relative humidity and a 12-h photoperiod for 5 weeks before bacterial and fungal inoculations. Tobacco (*Nicotiana benthamiana*) and tomato (*Solanum lycopersicum* cv Moneymaker) plants were grown on soil in a growth chamber (Convion) with 60% humidity and under a regime of 16-h light. Five-week-old plants were used in all the experiments.

### DNA Extraction and Genotyping

*gh3.2* (Col-0 background, salk\_037520C), *gh3.4* (salk\_102549C), and *gh3.6* (salk\_013458C) T-DNA insertion lines were provided by the Nottingham Arabidopsis Stock Centre (Alonso et al., 2003). Genomic DNA was isolated from two leaf discs for each T-DNA insertion line and for the corresponding wild-type plants. Seed material was segregated for the T-DNA insertions and homozygous lines for either mutant or wild-type alleles were identified using the PCR-based markers listed in Supplemental Table 1 online. *gh3.2* (Ler background), *gh3.5*, and *gh3.17* T-DNA insertion lines were characterized previously (Staswick et al., 2005; Zhang et al., 2007).

### Bacterial and Fungal Growth Conditions and Disease Assays

*Pseudomonas syringae* pv *tomato* DC3000 cultures were maintained and grown on Luria-Bertani medium containing rifampicin and kanamycin at 28°C. Cultures were centrifuged at 2500g for 10 min, and bacterial pellets were washed twice and resuspended in 10 mM MgCl<sub>2</sub> for plant inoculations.

The bacterial disease assays were performed as previously described (Katagiri et al., 2002). In brief, 5-week-old *Arabidopsis thaliana* plants were vacuum infiltrated with *P. syringae* DC3000 at 10<sup>6</sup> cfu/mL, and bacterial growth was monitored 0 and 3 DAI using serial dilution plating of ground leaf disks. Each time point represents the mean for nine 0.35-cm<sup>2</sup> leaf discs taken from three different plants.

The direct effect of IAA-Asp on the growth and expression of virulence genes of *Pst* DC3000 in vitro was studied as previously described (Vasil and Ochsner, 1999; Kim et al., 2009). The time points chosen for virulence gene expression are based on published reports (Vasil and Ochsner, 1999; Kim et al., 2009).

*Botrytis cinerea* was cultivated as previously described (El Oirdi et al., 2010). For gene expression and IAA-Asp quantification, 5-week-old plants sprayed with 5 × 10<sup>5</sup> spores/mL of *B. cinerea* were incubated in a growth chamber with high humidity. Water was used for mock treatments. *B. cinerea* disease assays were performed on detached leaves by measuring the disease area caused by the infection. Leaves of 5-week-old plants were detached and put into a Petri dish with the petiole embedded in solidified MS basal medium before inoculation with 5 µL of a 5 × 10<sup>5</sup> spores/mL suspension. Disease areas were measured at 2 DAI.

The direct effect of IAA-Asp on spore germination, germ tube growth, and virulence gene expression of *B. cinerea* was studied as previously described (Stefanato et al., 2009; El Oirdi et al., 2010).

### Plant Treatments

IAA, 2,4-D, and IAA-Asp were obtained from Sigma-Aldrich. They were first dissolved in ethanol and then diluted with distilled water to 10 mM stock solutions.

For experiments using *B. cinerea* as inoculum, plants were vacuum infiltrated with a solution containing 0.02% Silwet L-77 supplemented with

IAA, 2,4-D, or IAA-Asp at 50 or 100  $\mu\text{M}$ . Control plants were vacuum infiltrated with a solution containing ethanol and 0.02% Silwet L-77. Two hours later, plants were used for disease assays as described above.

For experiments using *P. syringae* DC3000, *Arabidopsis* plants were vacuum infiltrated with a solution containing 10 mM  $\text{MgCl}_2$ , 100  $\mu\text{M}$  IAA or IAA-Asp, and  $10^6$  cfu/mL of *P. syringae* DC3000. Control plants were vacuum infiltrated with a solution containing 10 mM  $\text{MgCl}_2$ , ethanol, and  $10^6$  cfu/mL of *P. syringae* DC3000. Similar experiments were done with *S. lycopersicum* cv Moneymaker and *N. benthamiana*, but the bacterial concentration was  $10^7$  cfu/mL and the infiltration was carried out with a syringe (Bouarab et al., 2002; Asai et al., 2008; Luo et al., 2009). The same concentration of IAA-Asp was tested, and control plants were syringe infiltrated with a solution containing 10 mM  $\text{MgCl}_2$ , ethanol, and  $10^7$  cfu/mL of *P. syringae* DC3000.

### Amino Acid-Conjugated IAA Quantification

Amino acid-conjugated IAAs were extracted and analyzed using ultraperformance liquid chromatography coupled with electrospray ionization tandem mass spectrometry as described previously (Chiwocha et al., 2003, 2005).

### Fig22 Responses Assays

Seedling growth inhibition was assessed as previously described (Zeng and He, 2010). Briefly, Col-0 and *gh3.2* seeds were grown in liquid MS medium containing 1% Suc supplemented with 10  $\mu\text{M}$  Fig22 (Feldan BIO). Root length was measured and seedlings were weighed 10 d after treatment.

For Fig22-induced gene expression, 10 seeds of Col-0 and *gh3.2* were grown in liquid MS medium containing 1% Suc. Nine days later, medium was changed and supplemented 24 h later with 10  $\mu\text{M}$  Fig22. Samples were harvested at 0, 30, and 60 min after Fig22 treatment for RNA extraction and qPCR analysis. Gene targets used in this study were previously described (Asai et al., 2002).

### Camalexin Quantification

Camalexin was extracted from *B. cinerea*-inoculated plants as described previously with some modifications (Glazebrook and Ausubel, 1994; Beets and Dubery, 2011). Briefly, 1 g of ground leaves was heated in 2 mL of 80% HPLC-grade methanol for 5 min. Samples were centrifuged for 5 min at 17,000g, and the supernatants were transferred to 2-mL tubes. The pellets were resuspended in methanol and the extraction steps were repeated. Methanol was evaporated and the residues resuspended in 100  $\mu\text{L}$  of chloroform (minimum 99% molecular biology; Sigma-Aldrich). Concentrated extracts were separated on 0.2-mm thin-layer chromatography (TLC) silica gel plates (60 Å Partisil K6F with fluorescent indicator; Chromatographic Specialties) using ethyl acetate/hexane (9:1) as a mobile phase. Long-wave UV light (365 nm) was used to visualize camalexin based on its blue fluorescence, and a camalexin standard migrated in the same TLC was used. The blue autofluorescent bands were scraped from the TLC plates and extracted with 350  $\mu\text{L}$  of HPLC-grade methanol. These fractions were then used for HPLC analysis.

Authentic samples of camalexin were obtained from Jane Glazebrook (University of Minnesota). A stock solution (100  $\mu\text{g/mL}$ ) of camalexin was prepared to obtain a standard ranging between 0.1 and 40  $\mu\text{g/mL}$ . The sample fractions were analyzed using a high performance liquid chromatograph (1260 Infinity; Agilent Technology) equipped with an auto-sampler and fitted with a column (Zorbax SB-C18, 4.6  $\times$  150 mm, 3.5- $\mu\text{m}$ ; Agilent). The volume injected was 10  $\mu\text{L}$ . The column was eluted at a flow rate of 1 mL/min with acetonitrile (A) and water (B) (90% B to 10% A) for 2 min, by a gradient (2% B to 98% A) for 11 min, then by 98% A for 2 min, followed by a decrease to 10% A for 2 min and holding for 3 min. Under these conditions, the camalexin peak appeared at retention times of 10.37 min. Detection was based on UV absorbance at 318 nm.

### RT-PCR Analysis

Total RNA was extracted from leaves and *B. cinerea* samples using the RNeasy plant mini kit according to the manufacturer's recommendations (Qiagen). First-strand cDNA was synthesized from 2  $\mu\text{g}$  total RNA using Superscript II reverse transcriptase (Invitrogen). For *Pst* DC3000, total RNA was extracted using the RNeasy Bacteria Mini Kit according to the manufacturer's recommendations (Qiagen). RNAs were treated with RNase-free DNase. Reverse transcription was performed using a first-strand cDNA synthesis kit (GE Healthcare). Targets were amplified using primers described in Supplemental Table 1 online. The qPCR was performed using the Eva Green method according to the manufacturer's recommendations (Bio-Rad). Melting curves were determined using the dissociation curve software SDS 2.2.2 to ensure that only a single product was amplified. The ABI PRISM 7500HT sequence detection system (Applied Biosystems) was used to detect the amplification level and was programmed with an initial step of 10 min at 95°C followed by 40 cycles alternating between 15 s at 95°C and 1 min at 60°C. All reactions were run in technical triplicates for each biological replicate, and the average values were used for quantification. The relative quantification of target genes was determined using the  $\Delta\Delta$  cycle threshold (CT) method. Briefly, the threshold cycle (Ct) values of target genes were normalized to an endogenous control gene ( $\Delta\text{CT} = \text{Ct}_{\text{target}} - \text{Ct}_{\text{endogenous}}$ ) and compared with a calibrator ( $\Delta\Delta\text{CT} = \Delta\text{CT}_{\text{sample}} - \Delta\text{CT}_{\text{calibrator}}$ ). Relative expression (RQ) was calculated using the sequence detection system SDS 2.2.2 software (Applied Biosystems) and the formula  $\text{RQ} = 2^{-\Delta\Delta\text{CT}}$ .

*RpoD* and *16S* were used as endogenous controls for *Pst* DC3000 targets analyzed in vitro and in planta, respectively. Elongation factor b (*BcEFb*) was used as an endogenous control for *B. cinerea* targets studied in vitro and in planta. Finally, elongation factor 1 (*EF1*) was used as an endogenous control for plant targets. Primers are detailed in Supplemental Table 1 online. Bacterial and fungal primers were tested with cDNA from noninfected versus infected plants to verify that they do not amplify cDNA from the plant. In all cases, no amplification was observed when cDNA from a noninfected plant was used, while amplification could be detected using cDNA from an infected plant. These results show that the bacterial and fungal primers specifically amplify cDNA from the pathogen. RT-PCR was done to show the transcript level of *GH3.2* in salk\_037520C, of *GH3.4* in salk\_102549C, and of *GH3.6* in salk\_013458C. Col-0 wild type was used for comparison. Primers used are described in Supplemental Table 1 online.

### Statistical Analysis

Statistical analysis was carried out using the GLM procedure of the SAS 9.1 statistical package. Data were subjected to either one- or two-way analysis of variance (ANOVA) depending on the experiment, followed by a comparison of the means according to a Duncan's multiple range test at  $P < 0.05$  or  $P < 0.01$ , unless otherwise stated. Pairwise comparisons with lower numbers of treatments were conducted according to Student's *t* test and Tukey's studentized range test (honestly significant difference [HSD]) at  $*P < 0.05$ ,  $**P < 0.01$ , or  $***P < 0.001$ .

### Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under the accession numbers in Supplemental Table 1 online.

### Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Figure 1.** IAA-Asp Enhances Disease Caused by *Pst* DC3000 in Tomato and Tobacco.

**Supplemental Figure 2.** Expression of Different *GH3* Genes Known to Conjugate Asp to IAA.

**Supplemental Figure 3.** Characterization of the Three Novel GH3 T-DNA Insertion Lines Used in This Study.

**Supplemental Figure 4.** Resistance to *B. cinerea* and *Pst* DC3000 Observed in *gh3.2* Is Not Mediated by JA and SA Signaling and Camalexin Pathways.

**Supplemental Figure 5.** IAA-Asp Does Not Affect the in Vitro Growth and Expression of Some Virulence Genes of *Pst* DC3000 and *B. cinerea*.

**Supplemental Table 1.** Primers Used in This Study.

## ACKNOWLEDGMENTS

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## AUTHOR CONTRIBUTIONS

R.G.-L. initiated this study. R.G.-L., M.E.O., N.B., and K.B. designed experiments, analyzed the data, and wrote the article. R.G.-L. and M.E.O. performed the experiments. N.B. and K.B. directed the study. N.B. and K.B. contributed equally to this work.

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# The Conjugated Auxin Indole-3-Acetic Acid–Aspartic Acid Promotes Plant Disease Development

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