Bacterial Effector Activates Jasmonate Signaling by Directly Targeting JAZ Transcriptional Repressors

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Abstract

Gram-negative bacterial pathogens deliver a variety of virulence proteins through the type III secretion system (T3SS) directly into the host cytoplasm. These type III secreted effectors (T3SEs) play an essential role in bacterial infection, mainly by targeting host immunity. However, the molecular basis of their functionalities remains largely enigmatic. Here, we show that the *Pseudomonas syringae* T3SE HopZ1a, a member of the widely distributed YopJ effector family, directly interacts with jasmonate ZIM-domain (JAZ) proteins through the conserved Jas domain in plant hosts. JAZs are transcription repressors of jasmonate (JA)-responsive genes and major components of the jasmonate receptor complex. Upon interaction, JAZs can be acetylated by HopZ1a through a putative acetyltransferase activity. Importantly, *P. syringae* producing the wild-type, but not a catalytic mutant of HopZ1a, promotes the degradation of HopZ1-interacting JAZs and activates JA signaling during bacterial infection. Furthermore, HopZ1a could partially rescue the virulence defect of a *P. syringae* mutant that lacks the production of coronatine, a JA-mimicking phytotoxin produced by a few *P. syringae* strains. These results highlight a novel example by which a bacterial effector directly manipulates the core regulators of phytohormone signaling to facilitate infection. The targeting of JAZ repressors by both coronatine toxin and HopZ1 effector suggests that the JA receptor complex is potentially a major hub of host targets for bacterial pathogens.


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Introduction

A prevailing concept for plant-pathogen interactions highlights the continuing battles between the activation of plant immune responses upon pathogen perception and the subversion of host immunity by virulence factors produced by successful pathogens. One branch of the plant immunity system is based on the recognition of pathogen- or microbe-associated molecular patterns (PAMP/MAMPs), which leads to a signal transduction cascade, and eventually PAMP-triggered immunity (PTI) [1]. PTI, broadly referred as basal defense in plants, restricts the growth of the vast majority of potential pathogens encountered by plants in the surrounding environment [2,3]. However, successful pathogens produce virulence factors to effectively suppress PTI. For example, Gram-negative bacterial pathogens, such as *Pseudomonas syringae*, inject type III-secreted effectors (T3SEs) into the host cell to actively inhabit PTI [4,5]. As a counter-attack strategy, plants have evolved nucleotide-binding leucine-rich repeat (NB-LRR) proteins to perceive specific T3SEs, directly or indirectly, and elicit effectortriggered immunity (ETI), which is often associated with localized programmed cell death at the infection sites [2,3,6].

Recent studies suggest that many *P. syringae* T3SEs suppress PTI and/or ETI by targeting important components of plant immunity [5,7,8]. Although the virulence targets of a few T3SEs have been characterized, the molecular mechanisms by which the majority of T3SEs subvert host resistance or facilitate nutrient acquisition remain elusive. HopZ1 is a *P. syringae* T3SE that belongs to the widely distributed YopJ family of cysteine proteases/acyltransferases produced by both plant and animal bacterial pathogens [9]. The YopJ-like T3SEs share a conserved catalytic core, consisting of three key amino acid residues (histidine, glutamic acid, and cysteine), which is identical to that of clan-CE (C55-family) cysteine proteases [10]. However, several members of the YopJ effector family have been shown to possess acetyltransferase activity. YopJ and VopA modify their target proteins (mitogen-associated protein kinases and Ikkβ/β) in animal hosts and the acetylation of these host targets blocks their phosphorylation and the subsequent defense signal transduction [11,12]. PopP2 produced by the plant pathogen *Ralstonia solanacearum* has an autoacetylation activity, which is essential for its recognition in resistant plants; however, whether PopP2 can modify its target proteins in the host remains unknown [13].

Two functional HopZ1 alleles, HopZ1a and HopZ1b, have been identified in *P. syringae* [9]. HopZ1b is produced by *P. syringae* pv. *glycinea* (Pgg) strains, which are the causative agents of bacterial
Author Summary

Many Gram-negative bacterial pathogens rely on the type III secretion system, which is a specialized protein secretion apparatus, to inject virulence proteins, called effectors, into the host cells. The type III secreted effectors (T3SEs) directly target host substrates in order to promote bacterial colonization and disease development. Therefore, the identification and characterization of the direct host targets of T3SEs provides important insights into virulence strategies employed by bacterial pathogens to cause diseases. Here, we report that the plant pathogen Pseudomonas syringae T3SE HopZ1a physically interacts with and modifies the jasmonate ZIM-domain (JAZ) proteins in plant hosts. JAZ proteins are components of the receptor complex of the plant hormone jasmonates (JA) and key transcription repressors regulating JA-responsive genes. HopZ1a belongs to the closely-related HopZ1a, αν2, HopZ1a in P. syringae pv. syringae strain A2; hereafter HopZ1a) triggers an HR in soybean cultivar Williams 82 and Arabidopsis thaliana accession Columbia-0 (Col-0, wild-type) [14]. HopZ1a mutants with the catalytic cysteine residues (C216 in HopZ1a or C212 in HopZ1b) substituted by alanines lose the virulence function or the HR-triggering activity, indicating that the functions of HopZ1 alleles require their enzymatic activities [9,14]. In addition, HopZ1 has a potential N-terminal myristoylation site (Gly2) which directs the proteins to the plasma membrane [14,15]. This myristoylation site of HopZ1a contributes to its virulence function in both soybean and Arabidopsis [14,15]. However, it is not clear whether this myristoylation site is important for the virulence function of HopZ1a. HopZ1a exhibited weak cysteine protease activity and could use tubulin as a substrate in vitro [9]. Recent studies showed that HopZ1a also possessed an acetyltransferase activity and could use tubulin as a substrate in vitro. Modification of tubulin is associated with the disruption of microtubule cytoskeleton, which may contribute to bacterial pathogenesis [16].

To identify potential host targets of HopZ1, we conducted yeast two-hybrid screening using a cDNA library of the natural host soybean and identified several HopZ1-interacting proteins (ZINPs). ZINP1 (2-hydroxyisovavanol dehydrogenase, GmHID1) is a key enzyme in the soybean isoflavone biosynthetic pathway and a positive regulator of soybean basal defense. HopZ1 induces the degradation of GmHID1, and hence a decreased isoflavone production in soybean, resulting in increased plant susceptibility to bacterial infection [17]. HopZ1 also enhances bacterial infection in Arabidopsis, which does not have a putative ortholog of GmHID1. To understand the mechanisms underlying the virulence function of HopZ1a in Arabidopsis, we characterized another family of ZINPs, which were identified as jasmonate ZIM-domain (JAZ) proteins. JAZs are key transcriptional repressors of the jasmonate (JA) signaling pathway and major components of the JA receptor complex [18,19,20]. JA plays an important role in regulating plant responses to biotic and abiotic stresses. Some P. syringae strains produce the JA-mimicking phytohormone coronatine, which efficiently activates JA signaling to facilitate bacterial entry into plant apoplastic space and suppress defense [21,22,23]. Therefore, HopZ1a may also target the JAZ proteins to promote bacterial infection. Consistent to this hypothesis, HopZ1a was previously reported to induce the expression of the JA/ethylene marker gene APDF1.2 in Arabidopsis, indicating that it could activate JA/ethylene signaling [24].

Here, we report that HopZ1a directly interacts with JAZ proteins of soybean and Arabidopsis. We show that HopZ1a induces the degradation of AtJAZ1, and promotes JA-responsive gene expression during P. syringae infection. Furthermore, HopZ1a functionally complements the growth deficiency of a P. syringae pv. tomato mutant that does not produce coronatine. All these activities depend on the intact catalytic core of HopZ1a, which acetylates JAZ proteins in vitro. Taken together, our results suggest that HopZ1a facilitates bacterial infection by manipulating the JA signaling pathway in Arabidopsis.

Results

HopZ1a physically interacts with GmJAZ1

Using yeast two-hybrid screens, we identified the HopZ1a-interacting proteins (ZINPs) from a soybean cDNA library [17]. Among them, ZINP3 (Gm7g04630) was interesting because it shows significant homology to the Jasmonate ZIM-domain (JAZ) proteins. We designated ZINP3 as GmJAZ1 because it is most similar (51% similarity in full-length amino acid sequences and 62% similarity in the ZIM and Jas domains) to AtJAZ1 in Arabidopsis. GmJAZ1 was then further pursued as a direct target of HopZ1a.

We first confirmed the physical interaction between HopZ1a and GmJAZ1 by in vitro pull-down using recombinant GST-HopZ1a and GmJAZ1-HA proteins over-expressed in E. coli. GST-HopZ1a or GST (empty vector) was purified from whole cell lysate using glutathione resins and then incubated with an equal amount of whole cell lysate of E. coli expressing GmJAZ1-HA. GST-HopZ1a-bound resins, but not GST-bound resins, provided enrichment of GmJAZ1-HA (Fig. 1A), suggesting that HopZ1a interacted with GmJAZ1 in vitro. The catalytic mutant HopZ1a(C216A) also interacted with GmJAZ1, similar to wild-type HopZ1a (Fig. 1A).

We next examined the sub-cellular localization of GmJAZ1 to determine whether it co-localizes with HopZ1a in plant cells. GmJAZ1-YFP was expressed in Nicotiana benthamiana using Agrobacterium-mediated transient expression. Yellow fluorescence was examined in the pavement cells of the infiltrated leaves at 48 hours post inoculation (hpi) using confocal microscopy. Fluorescence was detected both on the plasma membrane and in the nucleus (Fig. S1). Previous studies reported that HopZ1a mainly locates on the plasma membrane with a sub-pool of HopZ1a in the nucleus [17]. These results suggest that GmJAZ1 and HopZ1a could co-localize in plant cells. To further confirm that HopZ1a indeed enters the nucleus, we performed nuclear fractionation of N. benthamiana cells expressing HopZ1a(C216A). The catalytic mutant HopZ1a(C216A) was used in this experiment, because the expression of the functional HopZ1a triggers cell death in N. benthamiana [9,14]. Consistent with the previous confocal microscopy data [17], we detected the presence of HopZ1a(C216A) from both cytosolic and nuclear fractions (Fig. S2). These data confirmed that HopZ1a and GmJAZ1 co-localize in N. benthamiana cells.
proteins were expressed in E. coli. Precipitation of GmJAZ1 with HopZ1a was determined by western blots before (Input) and after affinity purification (Pull-down) using anti-HA antibody. The protein abundances of GST, GST-HopZ1a and GST-HopZ1a(C216A) on the affinity resins after washes were detected by Coomassie blue staining. (B) Bimolecular fluorescence complementation analysis showing HopZ1a-GmJAZ1 interactions in plant cells. HopZ1a(C216A)-nYFP and GmJAZ1-cYFP were co-expressed in N. benthamiana using Agrobacterium-mediated transient expression. Leaves co-infiltrated with Agrobacterium carrying GmJAZ1-cYFP or cYFP and HopZ1a(C216A)-nYFP were used as negative controls. Fluorescence was detected by confocal microscopy from the infiltrated tissues at 48 hpi. DAPI was used to stain the nuclei. These experiments were repeated three times with similar results.

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We further used the bimolecular fluorescence complementation (BiFC) assay to determine the interaction between HopZ1a and GmJAZ1 in planta. HopZ1a(C216A) and GmJAZ1 were fused to the nonfluorescent N-terminal domain of YFP (1–155 aa, nYFP) and the C-terminal domain of YFP (156–239 aa, cYFP), respectively, at their C-termini. When the fusion genes were co-expressed in N. benthamiana, fluorescence was detected on the plasma membrane and in the nucleus (Fig. 1B), consistent with the subcellular localization of GmJAZ1 and HopZ1a. Taken together, these experiments demonstrate the interaction of HopZ1a and GmJAZ1 in vitro and in planta.

**Interaction with HopZ1a leads to the degradation of GmJAZ1**

We have previously observed HopZ1-mediated degradation of another HopZ1-interacting protein GmHID1 when GmHID1 and HopZ1 were transiently co-expressed in N. benthamiana [17]. Therefore, we examined whether HopZ1a can also induce the degradation of GmJAZ1. GmJAZ1-FLAG and HopZ1a-HA were co-expressed in N. benthamiana, and the abundance of GmJAZ1 was determined at 20 hpi before the onset of visible cell death symptoms, which usually starts at 30 hpi. We chose 20 hpi because the expression level of GmJAZ1 was too low for protein analysis at earlier time points. A significant reduction of GmJAZ1 protein level was observed in N. benthamiana leaves co-expressing wild-type HopZ1a-HA, compared to leaves expressing the empty vector (Fig. 2A). These results suggest that HopZ1a induces the degradation of GmJAZ1 in plant cells and the degradation requires the enzymatic activity of HopZ1a.

Incubation of GmJAZ1 and HopZ1a proteins purified from E. coli did not lead to observable changes in the abundance of GmJAZ1 (Fig. 1A). We suspected that a plant factor(s) might be required for this process and therefore performed a semi-in vitro degradation assay by incubating proteins extracted from N. benthamiana tissues expressing GmJAZ1 or HopZ1a individually. Total proteins extracted from leaves expressing GmJAZ1 or HopZ1a were mixed and incubated at 4°C for six hours before the abundance of GmJAZ1 was examined using western blots. Again, a significant decrease in GmJAZ1 protein level was observed in the presence of wild-type HopZ1a, but not the catalytic mutant HopZ1a(C216A) [Fig. 2B]. These data suggest that HopZ1a induces GmJAZ1 degradation in plant cells.

To exclude the possibility that the reduced GmJAZ1 protein levels might have been resulted from cell death triggered by wild-type HopZ1a in N. benthamiana, we performed two control experiments. Firstly, we co-expressed the green fluorescence protein (GFP) with HopZ1a-HA or HopZ1a(C216A)-HA in N. benthamiana. The GFP protein levels remained unchanged in the presence of either wild-type or the catalytic mutant of HopZ1a (Fig. S3A). Secondly, we performed the semi-in vitro degradation assay of GmJAZ1 using AvrRpt2, which also elicits cell death in N. benthamiana [25]. Incubation with plant protein extracts expressing AvrRpt2 did not change the abundance of GmJAZ1 (Fig. S3B). This suggests that the reduced abundance of GmJAZ1 was not a result of HopZ1a-induced cell death in N. benthamiana.

**HopZ1a physically interacts with Arabidopsis JAZs**

Because GmJAZ1 is an ortholog of Arabidopsis JAZ proteins (AtJAZs), we examined whether HopZ1a also targets AtJAZs. Arabidopsis produces twelve JAZ orthologs (Fig. S4). Among them, seven were tested for their interactions with HopZ1a using in vitro pull-down. Our data showed that AtJAZ2, AtJAZ5, AtJAZ6, AtJAZ8 and AtJAZ12 interacted with HopZ1a in vitro (Fig. 3A). Although AtJAZ1 shares the highest sequence similarity with GmJAZ1 (Fig. S4), the interaction of AtJAZ1 with HopZ1a could not be determined because we were unable to express AtJAZ1 in E. coli at a level suitable for the pull-down assay.

We next confirmed the interaction between HopZ1a and AtJAZ6 in planta using BiFC. Similar to HopZ1a-GmJAZ1 interaction, yellow fluorescence was observed from plasma membrane and nucleus in cells co-expressing HopZ1a(C216A)-nYFP and AtJAZ6-cYFP (Fig. 3B). AtJAZ6 by itself was mainly located in the nucleus, but could also be detected in cytosol (Fig. S2). These data suggest that HopZ1a and AtJAZ6 co-localize and interact in plant cells.
HopZ1a acetylates JAZs in vitro

Several effectors from the YopJ family, including HopZ1a, have been shown to possess acetyltransferase activities. To determine whether JAZs are substrates of HopZ1a, we performed in vitro enzymatic assay using C14-labeled acetyl-CoA. Recombinant HIS-SUMO-HopZ1a or HIS-SUMO-HopZ1a(C216A) proteins were expressed in *E. coli* and purified using nickel column. The HIS-SUMO tag was then removed by ubiquitin like protease 1 (ULP1). Tag-free HopZ1a or HopZ1a(C216A) proteins were incubated with purified HIS-GmJAZ1 or MBP-AtJAZ6-HIS proteins in the presence of the cofactor inositol hexakisphosphate (IP6), and the acetylation of HopZ1a, GmJAZ1 and AtJAZ6 was detected by autoradiography as previously described [26]. Our experiments showed that both GmJAZ1 (Fig. 4A) and AtJAZ6 (Fig. 4B) were acetylated by wild-type HopZ1a, which also exhibited autoacetylation. The acetylation of GmJAZ1 appeared to be weaker in the autoradiograph compared to that of AtJAZ6. This is due to the low expression level of GmJAZ1 in *E. coli*, which only allowed us to use a much lower amount (1 µg), compared to AtJAZ6 (10 µg) in the reactions. Nonetheless, we consistently detected the acetylated form of GmJAZ1 when it was incubated with HopZ1a, but not HopZ1a(C216A), suggesting that GmJAZ1 and AtJAZ6 are both substrates of HopZ1a.

We sometimes could observe a background level of acetylation in tagged AtJAZ6 (MBP-AtJAZ6-HIS) when it was incubated with HopZ1a(C216A). Although this background acetylation was very weak compared to the acetylation of MBP-AtJAZ6-HIS, we decided to use the tag-free AtJAZ6 proteins to further confirm its acetylation by HopZ1a. Again, we observed strong acetylation of AtJAZ6 by HopZ1a, but not by HopZ1a(C216A) using only 5 µg of AtJAZ6 in the reaction (Fig. S5). These results demonstrate that GmJAZ1 and AtJAZ6 are acetylation substrates of HopZ1a.

The Jas domain of AtJAZ6 is required for HopZ1a interaction

JAZ proteins share three conserved domains: the C-terminal Jas motif [27], the ZIM domain in the central region [28], and a weakly conserved N-terminal region [19]. Because the conserved
Jas domain is essential for the instability of JAZs in response to JA and the JA-mimicking phytotoxin coronatine [18,19], we examined the impact of the Jas domain in the interaction between JAZs and HopZ1a. We constructed the mutant AtJAZ6\textsubscript{D Jas} by deleting ten highly conserved amino acids (from serine\textsubscript{191} to lysine\textsubscript{200}) within the Jas domain. In vitro pull-down assay showed that AtJAZ6\textsubscript{D Jas} did not bind HopZ1a (Fig. 4C). Furthermore, AtJAZ6\textsubscript{D Jas} was not acetylated by HopZ1a in vitro (Fig. 4D) or degraded by HopZ1a when these two proteins were co-expressed in \textit{N. benthamiana} (Fig. 4E). These results demonstrate that HopZ1a-induced JAZ degradation requires direct interaction of HopZ1a with AtJAZ6, which is mediated by the Jas domain.

HopZ1a triggers the degradation of AtJAZ1 during bacterial infection

Although we observed the degradation of GmJAZ1 and AtJAZ6 when they were co-expressed with HopZ1a in \textit{N. benthamiana}, it is important to examine whether HopZ1a can promote JAZ degradation during bacterial infection. For this purpose, we inoculated transgenic \textit{Arabidopsis} plants expressing 35S-HA-AtJAZ6
with *P. syringae* producing HopZ1a or HopZ1a(C216A). The *Arabidopsis* pathogen *Pseudomonas syringae* pv. *tomato* strain DC3000 (*PtoDC3000*) is well-known to induce AtJAZ degradation through the production of coronatine, which acts as a JA mimic [22]. The mutant *PtoDC3118* is deficient in coronatine production and therefore no longer degrades JAZs [29]. Importantly, *PtoDC3118* expressing HopZ1a from its native promoter also significantly reduced the abundance of AtJAZ1 at 6 hpi (Fig. 5A). The level of AtJAZ1 remained unchanged in tissues infiltrated with *PtoDC3118* carrying the empty vector or expressing the catalytic mutant HopZ1a(C216A). These data strongly suggest that HopZ1a can induce AtJAZ1 degradation during bacterial infection.

Because HopZ1a elicits HR in *Arabidopsis* ecotype Col-0, we performed two experiments to exclude the possibility that HopZ1a-triggered AtJAZ1 degradation was a result of plant cell death. First, we examined whether another effector AvrRpt2 could induce AtJAZ1 degradation. Although AvrRpt2 also triggers HR in *Arabidopsis* Col-0, the abundance of AtJAZ1 was unchanged when the HA-AtJAZ1-expressing plants were inoculated with *PtoDC3118* expressing AvrRpt2 (Fig. 5A). Next, we generated the transgenic *Arabidopsis* line expressing 35S-HA-AtJAZ1 in the *zar1-1* mutant background, which is abrogated in HopZ1a-triggered HR [30]. Again, the AtJAZ1 protein level was significantly reduced by HopZ1a (Fig. 5B), confirming that HopZ1a delivered by *P. syringae* leads to AtJAZ1 degradation in a cell death independent manner.

**HopZ1a-mediated JAZ degradation is dependent on coronatine-insensitive 1 (COI1)**

A major regulatory mechanism of JAZs in the presence of JA or coronatine is through COI1-dependent ubiquitin-proteasome degradation. COI1 is an F-box protein that determines the substrate specificity of a Skp/Cullin/F-box (SCF) E3 ubiquitin ligase-SCFCOI [31]. Together with JAZ, COI1 is also a critical component of the JA co-receptor complex [19,20,22]. We examined whether COI1 is required for HopZ1a-induced JAZ degradation using the transgenic *Arabidopsis* line expressing 35S-HA-AtJAZ1 in the col-10 (*SALK_035548*) mutant background. As expected, *PtoDC3000*, which induces JAZ degradation through coronatine production, was unable to reduce the abundance of AtJAZ1 in the absence of COI1. Interestingly, *PtoDC3118* expressing HopZ1a also no longer induced the degradation of AtJAZ1 in the col-1 mutant plants (Fig. 5C). These data suggest that, similar to coronatine- and JA-mediated AtJAZ degradation, COI1 is required for the degradation of AtJAZ1 by HopZ1a.

**HopZ1a activates JA signaling**

In *Arabidopsis*, JAZ proteins are repressors of JA transcription factors (e.g. AtMYC2) that are involved in the expression of JA-responsive genes [32,33,34]. Since HopZ1a induces the degradation of AtJAZ1, we examined whether it could induce the expression of JA-responsive genes in *Arabidopsis*. Five-week-old *zar1-1* plants were inoculated with *PtoDC3118* expressing HopZ1a or HopZ1a(C216A) at OD600 = 0.2 (approximately 2 × 10⁸ cfu/mL). The transcript levels of two early JA-responsive genes, *AtJAZ9* and *AtJAZ10* [34], were analyzed at 6 hpi. Both genes were induced approximately ten fold in plants infected by *PtoDC3118* (HopZ1a), whereas their expression was not changed in tissues infected by

![Figure 5. HopZ1a triggers the degradation of AtJAZ1 during bacterial infection.](image)

**Figure 5. HopZ1a triggers the degradation of AtJAZ1 during bacterial infection.** (A) HopZ1a, but not AvrRpt2, promotes the degradation of AtJAZ1 in the *Arabidopsis* ecotype Col-0 (wild-type) during bacterial infection. Six-week-old 35S-HA-AtJAZ1 Arabidopsis transgenic plants were infiltrated with *PtoDC3000*, *PtoDC3118* carrying the empty pUCP18 vector (EV), or *PtoDC3118* expressing HopZ1a, HopZ1a(C216A) or AvrRpt2. (B) HopZ1a promotes the degradation of AtJAZ1 in the *zar1-1* Arabidopsis plants. Six-week-old 35S-HA-AtJAZ1 Arabidopsis plants were inoculated with *PtoDC3000*, *PtoDC3118* carrying the empty pUCP18 vector (EV), or *PtoDC3118* expressing HopZ1a or HopZ1a(C216A). (C) HopZ1a-mediated degradation of AtJAZ1 is dependent on COI1. 35S-HA-AtJAZ1, coi1-30 Arabidopsis plants were inoculated with *PtoDC3000*, *PtoDC3118* carrying the empty pUCP18 vector (EV), or *PtoDC3118* expressing HopZ1a or HopZ1a(C216A). Bacterial infection assays were conducted using inoculums at OD600 = 0.2 (approximately 2 × 10⁸ cfu/mL). The abundance of AtJAZ1 was determined by western blots using anti-HA antibody at 6 hpi. The protein gels were stained with Coomassie blue as loading controls. These experiments were repeated three times with similar results.
PhDC3118 expressing HopZ1a(C216A) (Fig. 6A). The level of gene induction by HopZ1a was lower than that by coronatine, as shown by the approximately 40-fold induction of AtJAZ9 and AtJAZ10 in plants infected with PhDC3000. This is consistent with the partial vs. complete degradation of AtJAZ1 by HopZ1a or coronatine during bacterial infection. Nonetheless, these experiments suggest that bacterium-delivered HopZ1a can activate JA signaling.

Recent findings showed that coronatine can suppress salicylic acid (SA) accumulation, probably as a consequence of the activation of JA signaling [35]. Because SA-associated defense confers resistance against biotrophic and hemibiotrophic pathogens, reduced SA accumulation would lead to defense suppression. In particular, coronatine is able to repress the expression of the SA synthetic enzyme isochorismatase synthase gene 1 (AtICS1) in Arabidopsis. We then examined the impact of HopZ1a on the expression of AtICS1. Arabidopsis zar1-1 mutant plants were inoculated with PhDC3000 or PhDC3118 carrying empty vector, HopZ1a, or HopZ1a(C216A) at OD₆₀₀ = 0.2 (approximately 2 × 10⁸ cfu/mL). Consistent with the prior findings, the transcript abundance of AtICS1 was reduced in plants infected with PhDC3000 when compared to PhDC3118 carrying the empty vector or HopZ1a(C216A) (Fig. 6B). The expression of AtICS1 was also reduced in plants inoculated with PhDC3118 expressing HopZ1a, to a similar level as that in leaves inoculated with PhDC3000. These data confirmed that, like coronatine, HopZ1a activates JA signaling and represses SA accumulation during bacterial infection.

HopZ1a facilitates bacterial multiplication in Arabidopsis in a COI1-dependent manner

Coronatine facilitates the infection of PhDC3000 by activating JA signaling in Arabidopsis [21]. The coronatine-deficient mutant PhDC3118 exhibits a significant reduction in bacterial population especially when the plants are infected by dipping inoculation [36]. Since HopZ1a also activates JA signaling, we examined whether HopZ1a could complement the growth deficiency of PhDC3118. The Arabidopsis zar1-1 mutant plants were dipping-inoculated by PhDC3000 or PhDC3118 carrying the empty vector, HopZ1a, or HopZ1a(C216A). Three days post infection (dpi), the bacterial populations of PhDC3118 carrying the empty vector or expressing HopZ1a(C216A) were approximately 200 fold lower than that of PhDC3000 (Fig. 7A). Importantly, PhDC3118 expressing wild-type HopZ1a multiplied to a significantly higher level (about 10 fold) than PhDC3118 or PhDC3118 expressing HopZ1a(C216A) (Fig. 7A). Although the population of PhDC3118(HopZ1a) is lower than that of PhDC3000, this partial complementation of the growth deficiency of PhDC3118 is consistent with the partial degradation of AtJAZ1 (Fig. 5A) and the lower levels of JA-responsive gene induction (Fig. 6A) by PhDC3118(HopZ1a) compared to PhDC3000.

To further confirm that the function of HopZ1a is specifically related to its ability to activate the JA pathway, we introduced HopZ1a into PhDC3000 and performed the same bacterial growth assay. HopZ1a was previously shown to enhance the infection of PhDC3000 [30]. However, despite numerous trials, we did not observe any enhancement of HopZ1a on in planta multiplication of PhDC3000. In fact, we consistently detected a decrease in the population of PhDC3000(HopZ1a) compared to PhDC3000 carrying the empty vector (Fig. 7A). Thus, HopZ1a can partially substitute for coronatine to promote bacterial infection.

Because COI1 is required for HopZ1a-induced degradation of AtJAZ1, we then examined whether COI1 is also required for the virulence activity of HopZ1a in Arabidopsis. For this purpose, we generated coi1-1, zar1-1 double mutant Arabidopsis plants, which were inoculated by PhDC3118 carrying the empty vector, HopZ1a or HopZ1a(C216A). The bacterial populations of these three strains remained the same (Fig. 7B), confirming that the virulence activity of HopZ1a requires COI1. To further confirm that HopZ1a was unable to activate JA signaling in the mutant plants, we also determined the transcript levels of the JA-responsive genes AtJAZ9 and AtJAZ10, as well as the SA-biosynthetic gene AtICS1 after bacterial inoculation. Similar to PhDC3000, PhDC3118 expressing HopZ1a was also unable to induce the expression of AtJAZ9 and AtJAZ10 or suppress the expression of AtICS1 (Fig. 5B). These data suggest that both the phytoxotox coronatine and the effector HopZ1a activate JA signaling in a COI1-dependent manner.

**Figure 6. HopZ1a activates JA signaling during bacterial infection.** Arabidopsis zar1-1 mutant plants were inoculated with PhoDC3000 or PhoDC3118 carrying the empty pUCP18 vector (EV), HopZ1a or HopZ1a(C216A). The transcript levels of the JA-responsive genes AtJAZ9 and AtJAZ10, as well as the SA biosynthetic gene AtICS1 were determined by quantitative RT-PCR. (A) HopZ1a induces the expression of JA-responsive genes in Arabidopsis. The abundances of AtJAZ9 and AtJAZ10 transcripts were examined at 6 hpi using AtActin as the internal standard. Relative expression levels were determined by comparing the normalized AtJAZ9 or AtJAZ10 transcripts between infected and mock-treated (leaves infiltrated with 10 mM MgSO₄) samples. (B) HopZ1a reduces the expression of AtICS1 in Arabidopsis. AtICS1 transcript level was analyzed at 9 hpi using AtUBQ5 as the internal standard. Values are means ± standard deviations (as error bars) (n = 5). All experiments were repeated at least five times with similar results. The expression of HopZ1a in P. syringae was confirmed by western blots (Fig. S8). doi:10.1371/journal.ppat.1003715.g006

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HopZ1a partially complements the virulence function of coronatine in bacteria growth. (A) HopZ1a promotes the multiplication of P. syringae DC3118 in Arabidopsis. Arabidopsis zar1-1 plants were dip-inoculated with P. syringae DC3000 carrying pUCP20tk (EV), pUCP20tk-hopZ1a-HA, or P. syringae DC3118 carrying pUCP18 (EV), pUCP18:hopZ1a-HA or pUCP18:hopZ1a(C216A)-HA at OD600 = 0.2 (approximately 2 x 10^8 CFU/mL). Bacterial populations were determined at 0 and 3 days post inoculation. The average colony forming units per square centimeter (CFU/cm^2) and standard deviations (as error bars) are presented. Different letters at the top of the bars represent data with statistically significant differences (two tailed t-test p < 0.01). (B) COI1 is required for the virulence activity of HopZ1a. col1-1, zar1-1 double mutant plants were dip-inoculated with P. syringae DC3118 carrying pUCP18 (EV), pUCP18:hopZ1a-HA or pUCP18:hopZ1a(C216A)-HA. Bacterial multiplications were examined at 0 and 3 days post inoculation. The average colony forming units per square centimeter (CFU/cm^2) and standard deviations (as error bars) are presented. The expression of HopZ1a or HopZ1a(C216A) in P. syringae was confirmed by western blots (Fig. S8). These experiments were repeated at least five times with similar results. doi:10.1371/journal.ppat.1003715.g007

Discussion

T3SEs manipulate a variety of cellular processes in eukaryotic hosts for the benefit of pathogen infection. Emerging data suggest that bacterial pathogens have evolved various effectors to manipulate the signaling of JA and SA, which are important plant hormones that regulate defense response [37]. JA-dependent defense plays a major role in plant immunity against biotrophic and hemibiotrophic pathogens, such as Hyaloperonospora arabidopsidis and P. syringae [35,37]. The P. syringae effector HopI1 directly targets Hsp70 in chloroplasts to suppress SA accumulation and thereby facilitate bacterial infection [38]. In addition, the Xanthomonas campestris effector XopD was also shown to repress SA signaling during bacterial infection of tomato [39,40,41]. Here, we report that the P. syringae effector HopZ1a represses SA accumulation, probably as an indirect effect of the activation of JA signaling.

In this study, we report that HopZ1 directly targets JAZs, the key negative regulators of JA signaling [18,19]. Because JA signaling pathway is antagonistic to SA-dependent defense, activating JA signaling would be an attractive bacterial strategy to suppress host defense and facilitate pathogenesis of these pathogens. Indeed, recent studies have shown a remarkable example in which the P. syringae phytotoxin coronatine structurally mimics the active form of JA and targets the JAZ repressors for degradation to efficiently activate JA signaling [20,22]. However, it has been rather puzzling that only a few P. syringae strains produce coronatine [42]. A previous study showed that a T3SE, AvrB, was also able to promote JA signaling, apparently through an indirect mechanism via the activation of MPK4 [43,44]. We show here that the effector HopZ1a directly interacts with JAZs and at least some JAZs can be used by HopZ1a as substrates for acetylation. Importantly, HopZ1 mediates the degradation of AtJAZ1 in Arabidopsis and promotes bacterial infection in a COI1-dependent manner. This new finding raises the exciting possibility that JAZ repressors (hence the JA receptor complex) may be a common hub of host targets for diverse bacterial virulence factors. Consistent with this notion, oomycete pathogens were also found to produce effectors that interact with AtJAZ1 [45]. These pieces of evidence suggest that the JAZ receptor complex might be the Achilles’ heel in plant defense system during the arms race with microbial pathogens.

HopZ1a enhances the in planta multiplication of P. syringae DC3118, but not that of P. syringae DC3000, in Arabidopsis. A weak growth enhancement of P. syringae DC3000 by HopZ1a was reported previously [30]. We were unable to replicate the published data, probably due to differences in experimental conditions. Our experiments, including the JA-responsive gene expression, JAZ protein degradation and bacterial in planta multiplication, consistently suggest that HopZ1a activates the JA signaling pathway in a manner similar to coronatine. However, HopZ1a only partially complements the function of coronatine. This could be because HopZ1a is not as potent as coronatine in inducing the degradation of JAZs. Coronatine has dual functions during the pre-entry and post-entry stages of bacterial infection [23], whereas the type III secretion genes are generally believed to be expressed after the bacteria have entered the apoplast [46]. Although it remains to be determined whether HopZ1a could promote stomata opening at the pre-entry stage, it is possible that HopZ1a mainly substitutes coronatine function inside the plant tissues. In addition, proteins might not be as stable as metabolites in planta, which may also explain the partial complementation of HopZ1a for the virulence deficiency of the coronatine mutant PbDC3118.

As transcription regulators, JAZs are believed to function in the nucleus. However, HopZ1a was previously shown to mainly locate
Type II Effector Manipulates Jasmonate Signaling

on the plasma membrane and this localization was mediated by a potential myristoylation site in the N-terminus [15]. Our protein-protein interaction and localization analyses showed that HopZ1a is also located in the nucleus and it interacts with JAZs both in the nucleus and on the cytosol/plasma membrane. Importantly, the mutant HopZ1a(G2A), which is abolished for its localization on the plasma membrane, was still able to promote PtoDC3118 infection. These data demonstrate that the membrane localization of HopZ1a is only important for host recognition [14,15], but not required for virulence activity. This is consistent with the primary localization of JAZs in the nucleus as transcription repressors.

Hop1-like T3SSs produced by plant pathogens appear to have various enzymatic activities. AvrXv4 of Xanthomonas campestris was reported to be a SUMO protease [47]. AvrBsT, also from Xanthomonas, exhibited a weak cysteine protease activity in vitro [48]. PopP2 in R. solanacearum has autoacetylation and trans-acytilation activities in vitro, but it does not seem to acetylate its host target proteins [13]. Recently, HopZ1a was demonstrated to acetylate tubulin [16]. Our experiments showed that GmJAZ1 and AtJAZ6 are also substrates of HopZ1a. Importantly, we found that HopZ1a induces the degradation of AtJAZ1 during bacterial infection. In the presence of the active form of JA or coronatine, the F-box protein COI1 interacts with JAZs via the C-terminus Jas motif and recruits JAZs to the 26S proteasomes for degradation [18,19,22]. The fact that HopZ1a-mediated AtJAZ1 degradation is dependent on COI1 suggests that this degradation could also be dependent on the 26S proteasomes as a consequence of JAZ modification by HopZ1a. Post-translational modifications, including acetylation, have been shown to induce or repress proteasomal degradation. For example, in mammalian cells, the acetyltransferase ARD1 acetylates Hypoxia-inducible factor 1α (HIF-1α), which promotes its ubiquitination and proteasomal degradation [49]. Further investigations are needed to determine how HopZ1a-mediated acetylation of JAZs could facilitate COI1-dependent degradation of JAZ repressors.

Materials and Methods

Bacterial strains and plasmids

Pseudomonas syringae, Agrobacterium tumefaciens and Escherichia coli strains were grown as described [50]. Bacteria strains and plasmids used in this study are summarized in Table S1.

Fluorescence microscopy

To construct plasmids for bimolecular fluorescence complementation (BiFC) assay, full-length cDNA of GmJAZ1 or AtJAZ6 and hopZ1a in (C216A) were cloned into the vectors pSPYCE and pSPYNE [51], respectively. To examine the subcellular localization of GmJAZ1, full-length cDNA was cloned into the vector pEG101 [52]. The recombinant plasmids were introduced into Agrobacterium tumefaciens strain C58C1[pCH32], which were then used to infiltrate 3–4 week old Nicotiana benthamiana plants using a protocol described previously [14]. Functional fluorophore were visualized in the infiltrated leaves using a Leica SP2 Laser Scanning Confocal Microscope (Leica Microsystems) at 48 hpi for subcellular localization and BiFC. DAPI was used to stain the nucleus in plant cells [53,54].

In vitro GST pull-down assays

To construct GST-fusion plasmids, the full-length hopZ1a gene was inserted into the vector pGEX4T-2 (GE Healthcare Life Science). GmJAZ1-H1 gene was cloned into the vector pET14b (Navogen), which has 6×His in the N-terminus. The AtJAZ6 genes were cloned into the plasmid vector pET-Mal with maltose binding protein (MBP) in the N-terminus and 8×His in the C-terminus [55]. In vitro pull-down assays were carried out using GST pull-down protein:protein interaction kit (Pierce) according to the manufacturer’s instruction. Briefly, GST or GST-HopZ1a was expressed in E. coli strain BL21(DE3). Soluble proteins were incubated with 50 μL glutathione agarose beads (Invitrogen) for one hour at 4°C. The beads were washed (20 mM Tris-HCl (PH 7.5), 150 mM KCl, 0.1 mM EDTA and 0.05% Triton X-100) five times and then incubated with equal amount of bacterial lysates containing JAZ proteins at 4°C for overnight. The beads were washed five times again, and the presence of the JAZ proteins on the beads was detected by western blots using anti-HA or anti-His antibodies conjugated with horseradish peroxidase (HRP) (Santa Cruz Biotechnology Inc.).

Protein analysis

Pseudomonas syringae expressing the hopZ1a-HA proteins was induced in M63 minimal medium containing 1% fructose at room temperature overnight [50]. HopZ1a expression was detected by western blots using the anti-HA antibody.

For JAZ degradation assay in N. benthamiana, hopZ1a-HA or 3×FLAG-hopZ1a were co-expressed with GmJAZ1-FLAG or AtJAZ6-FLAG-HIS using Agrobacterium-mediated transient expression as previously described [9,56]. Leaf disks were collected at 20 hpi, and then grounded in 2×Laemmlli buffer. The abundances of GmJAZ1 and AtJAZ6 were analyzed by western blots.

For the semi-in vitro protein degradation assay, GmJAZ1-FLAG and 3×FLAG-HopZ1a were over-expressed individually in N. benthamiana using Agrobacterium-mediated transient expression. Total proteins were extracted from the infected leaf tissues at 20 hpi using an extraction buffer containing 200 mM NaCl, 50 mM Tris (pH 7.6), 10% Glycerol, 0.1% NP-40, protease inhibitor cocktail (Roche), 10 mM DTT, 1 mM PMSF. Protein extracts were mixed in equal volume for six hours at 4°C with gentle agitation before the abundance of GmJAZ1 was examined by western blots.

For the in vivo JAZ degradation assay, six-week-old 35S:HA-AfJAZ1 transgenic Arabidopsis plants were hand infiltrated with bacterial suspensions of PfDC3000 or PfDC3118 carrying the empty vector (EV), expressing HopZ1a, HopZ1a(C216A) or AvrRpt2 at OD600 = 0.2 (approximately 2×108 cfu/mL). Leaves infiltrated with water were used as a negative control. Six hours post inoculation, total proteins were extracted from four leaf discs (0.5 cm²) in 100 μL of 2×SDS sample buffer. The lysates were incubated at 95°C for 10 minutes followed by centrifugation at 14,000 rpm for 5 minutes. The abundance of AtJAZ1 was then analyzed by western blots. Homozygous coi1-30 mutant plants were selected on 1/2 Murashige & Skoog (MS) medium supplemented with 50 μM JA. Seedlings that were insensitive to JA treatment, i.e. without inhibited root growth symptoms, were transplanted in soil and infected with P. syringae after six weeks.

In vitro acetylation assays

HIS-GmJAZ1, HIS-SUMO-HopZ1a, HIS-SUMO-HopZ1a(C216A), HIS-SUMO-AfJAZ6, MBP-AfJAZ6-HIS, and MBP-AfJAZ6Δjas-HIS were over-expressed in the E. coli strain BL21(DE3) and then purified using nickel resins. HIS-SUMO-HopZ1a and HIS-SUMO-AfJAZ6 proteins were then cleaved by ULP1 protease, producing protein mixtures with both HIS-SUMO and either tag-free HopZ1a or AfJAZ6. The protein mixtures were incubated with nickel resin again and the tag-free HopZ1a or AfJAZ6 proteins were collected from the flow through. In a standard acetylation assay, 2 μg purified HopZ1a or HopZ1a(C216A) was incubated with 10 μg MBP-AfJAZ6, 5 μg
A5J6 or 1 μg GmJAZ1 at 30 °C for one hour in 25 μL of reaction buffer containing 50 mM HEPEs (pH 8.0), 10% glycerol, 1 mM DTT, 1 mM PMSE, 10 mM sodium butyrate, 1 μL [14C]-acetyl-CoA (55 μCi/μmol) and 100 mM IP6. The reaction mixtures were then subjected to SDS-PAGE and acetylated proteins were detected by autoradiography as previously described [11,26,37] after exposure at −80 °C for five days. After autoradiography, the protein gels were removed from the filter paper and stained with Coomassie blue as a loading control.

Real time RT-PCR

The transcript abundances of A5J69, A5J610 or AtICS1 in Arabidopsis leaf tissues were analyzed by real-time RT-PCR using SYBR Green Supermix (BioRad Laboratories) and an CFX96 Real-Time PCR Detection System (BioRad Laboratories). Total RNA was isolated from three independent biological replicates using Trizol, and DNA was removed using DNase I (Fermentas). Reverse transcription was performed using M-MLV Reverse Transcriptase (Promega) with 1 μg of total RNA in a 25 μL reaction. The cDNAs were then used as templates for real-time PCR using gene-specific primers, which are listed below. 

\[\text{AtUBQ5: } 5'\text{-GACGCTTCATCTCGTCC-3'} \text{ and } 5'\text{-CTAGCTTTACGATGATCGTGTG-3'}\]

\[\text{AtICS1: } 5'\text{-GTAGTTTCCGAGATATTTCAAGGTTG-3'} \text{ and } 5'\text{-GAAAGACAGGAGATTAGGCC-3'}\]

\[\text{AtUBQ5: } 5'\text{-GACGCTTCATCTCGTCC-3'} \text{ and } 5'\text{-GTAACGTTAGTGCTCC-3'}\]

\[\text{AtICS1: } 5'\text{-GGCCAGGGAACCTTACG-3'} \text{ and } 5'\text{-AGGTCC-CGCATACTTAC-3'}\]

In planta bacterial multiplication assays

Arabidopsis plants were planted as previously described [30,36]. The leaves of five-week old plants were dipped into the bacterial suspensions at an OD600 = 0.2 (approximately 2×109 cfu/mL) for 15 seconds. The inoculated plants were then transferred to a growth chamber (22 °C and 16/8 light/dark regime, 90% humidity). Bacterial populations were determined as colony forming units (cfu) per cm2 using a previously described procedure [11,26,37].

Statistical analysis

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

Supporting Information

Figure S1 Subcellular localization of GmJAZ1 in plant cells. GmJAZ1-YFP was transiently expressed in N. benthamiana and the fluorescence was observed at 48 hours post Agro-infiltration. DAPI was used to stain the nucleus. This experiment was repeated three times with similar results.

Figure S2 HopZ1a(C216A) localizes in both cytosol and nucleus. HopZ1a(C216A) and 3×HA-A5J6 were cloned into a T-DNA binary vector pHPl4-GW-Venus [Jian Yao and Sheng Yang He, unpublished] and pYP003 [58], respectively. HopZ1a(C216A)-YFP and 3×HA-A5J6 were co-expressed in N. benthamiana by Agrobacterium-mediated transient transformation.

Leaf tissues were collected at two days post infiltration and subjected to nuclear protein fractionation by using nuclear protein Extraction Kit (Sigma). Proteins from different fractions were detected by Western blots. Histone 3 and UDP are marker proteins that can be detected from nuclear and cytosolic fractions, respectively. Anti-GFP, anti-HA, anti-Histone 3 and anti-UDP antibodies were used to verify the expression of HopZ1a(C216A)-YFP, HA-A5J6 or marker proteins. This experiment was repeated twice with similar results.

Figure S3 HopZ1a-triggered GmJAZ1 degradation in N. benthamiana is independent of plant cell death. (A) GFP protein level was not altered when co-expressed with HopZ1a. GFP was under the control of CaMV 35S promoter and co-expressed in N. benthamiana with HopZ1a using Agrobacterium-mediated transient expression. The abundance of the GFP protein was determined using anti-GFP antibody at 24 hpi. Anti-HA antibody was used to verify the expression of the HopZ1a proteins. The protein gel was stained with Coomassie blue as a loading control. (B) AvrRpt2 did not induce GmJAZ1 degradation although it elicits cell death in N. benthamiana. GmJAZ1-FLAG and AvrRpt2-HA were transiently expressed in N. benthamiana individually. Total proteins were extracted from the infiltrated leaves at 20 hours post Agro-infiltration, mixed in equal volume, and incubated at 4 °C for six hours. The abundance of GmJAZ1-FLAG was then analyzed by western blots. The bands corresponding to AvrRpt2 were labeled with *. These experiments were repeated twice with similar results.

Figure S4 Phylogenetic analysis of GmJAZ1 and AtJAZs.

The PhyML tree was generated using full-length protein sequences by Seaview [59].

Figure S5 HopZ1a strongly acetylates tag-free A5J6.

Tag-free HopZ1a or HopZ1a(C216A), and A5J6 were purified from E. coli and co-infiltrated into N. benthamiana to verify their expression. This experiment was repeated twice with similar results.

Figure S6 HopZ1a no longer activates JA signaling in coil-1 mutant Arabidopsis.

Arabidopsis coil-1, coil-1 mutant plants were inoculated with PtoDC3000 or PtoDC3118 carrying the empty pUGP18 vector (EV), HopZ1a or HopZ1a(C216A). Relative expressions of A5J69, A5J610 or AtICS1 were determined by comparing the normalized transcript levels between the infected and the mock-inoculated samples (leaves infiltrated with 10 mM MgSO4). AtUBQ5 was used as the internal standard.

(A) Transcript abundances of the JA-responsive genes A5J69 and A5J610 were determined at 6 hpi. (B) Transcript level of AtICS1 was determined at 9 hpi. Values are means ± standard deviations (as error bars) (n = 3). All experiments were repeated twice with similar results.

Figure S7 HopZ1a(G2A) facilitates PtoDC3118 infection to the same extent as wild-type HopZ1a.

PtoDC3118 expressing the empty vector (EV), HopZ1a, HopZ1a(C216A) or HopZ1a(G2A) were used to dip-inoculate five-week old Arabidopsis coil-1 plants. Colony forming units (cfu) were determined at 0 day and 3 dpi. The average colony forming units per square centimeter (cfu/cm2) of four biological replicates are presented with error bars showing the standard deviations. Different letters at the top of the bars represent data with statistically significant differences.
differences (two tailed p<0.01). This experiment was repeated twice with similar results.

(DOC)

Figure S8 HopZ1a expression in PtoDC3000 or PtoDC3118 cells was demonstrated by western blots to confirm that differences in the cellular function of the wild type and the catalytic mutant of HopZ1a in planta were not due to different protein expression in P. syringae. Bacterial cells were induced in M63 minimal medium containing 1% fructose. Total proteins from equal amount of the induced cells were extracted and HopZ1a, HopZ1a(C216A) or HopZ1a(G2A) proteins were detected by western blots using anti-HA antibody. These experiments were repeated twice with similar results.

(DOC)

Table S1 Bacterial strains and plasmids used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
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<tr>
<td>PtoDC3000</td>
<td>Pseudomonas syringae pv. tomato DC3000</td>
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<tr>
<td>PtoDC3118</td>
<td>Pseudomonas syringae pv. tomato DC3118</td>
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References


