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Functional Study of HopZ1, a Type III Secreted Effector of the Bacterial Pathogen *Pseudomonas syringae*

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Shushu Jiang performed biological experiments as shown in Figures 1, 2, 3, 4, 5B, 5C, 6, 7A, S1, S3, S4, S5, S6, S7 and S8.
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Ka-Wai Ma performed experiment as shown in Figure 7B and generated *coi1-1, zar1-1* homozygous mutants.
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ABSTRACT OF THE DISSERTATION

Functional Study of HopZ1, a Type III Secreted Effector of the Bacterial Pathogen *Pseudomonas syringae*

by

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University of California, Riverside, December 2013
Dr. Wenbo Ma, Chairperson

Many Gram-negative phytopathogenic bacteria inject an array of type III secreted effectors (T3SEs) into plant cells via the type III secretion system (T3SS). These effectors play essential roles in bacterial infection mainly by targeting host immunity. However, the molecular basis of their functionalities remains largely unknown.

My research focuses on a T3SE produced by *Pseudomonas syringae*, called HopZ1, which is a member of the widely distributed YopJ effector family. Our lab identified several HopZ1 interacting proteins (ZINPs) from soybean using yeast two-hybrid screening, among which GmJAZ1 is homologous to *Arabidopsis* jasmonate ZIM-domain (AtJAZ) proteins. JAZs are key negative regulators of the jasmonate (JA) signaling and major components of the JA receptor complex. The main goal of my thesis is to investigate the function of HopZ1 in plant hosts by directly targeting the JAZ proteins.

In Chapter one, I proved that HopZ1 interacts with both GmJAZ1 and multiple AtJAZs in vitro and in planta. Upon interaction, JAZs can be acetylated by HopZ1a through a putative acetyltransferase activity. In addition, during bacterial infection of

Arabidopsis, *P. syringae* producing wild type, but not the catalytic mutant, of HopZ1a induces the degradation of JAZs, activates the expression of JA-responsive genes, and suppresses the salicylic acid (SA) signaling pathway. As a result, HopZ1a promotes *P. syringae* infection in *Arabidopsis*.

In Chapter two, I determined that HopZ1a can acetylate AtJAZ6, AtJAZ8, AtJAZ9 and AtJAZ10 in vitro. Mass spectrometry analyses suggest that multiple serine/threonine residues within the TSYDSDSSDTTS peptide in the N-terminal region of AtJAZ8 are potentially acetylated by HopZ1a. AtJAZ8 mutants with this sequence deleted, or with all the eight serine and threonine residues within this sequence substituted with alanines, are no longer acetylated by HopZ1a, suggesting that one or more serine/threonine residues within this sequence are the acetylation sites.

My research provides the first example of a bacterial effector that subverts host immunity by directly targeting the receptor complex of a defense-associated hormone in plants. This work also revealed a novel mechanism to regulate the JA signaling in plants through posttranslational modification of JAZs.

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General Introduction

Pathogen-associated molecular patterns (PAMPs) and plant basal defense

Plants are exposed to numerous microbes existing in the surrounding environment and are constantly attacked by various potential pathogens. In order to fight with those pathogens, plants evolved different levels of immune responses (Chisholm et al, 2006; Jones & Dangl, 2006). The patterns of the dynamic co-evolutionary arms race between plants and pathogens have been nicely summarized in the "Zig-Zag" model introduced by Jones and Dangl (Jones & Dangl, 2006). The first layer of plant innate immunity is believed to start with the recognition of pathogen "elicitors" by pattern-recognition receptors (PRRs), which are located at the plant cell surface (Zipfel, 2008). These "elicitors" produced by plant pathogens are termed pathogen-associated molecular patterns (PAMPs) or microbe-associated molecular patterns (MAMPs) (Ausubel, 2005). The PAMP-triggered plant basal defense is accordingly called *PAMP-triggered immunity* (PTI).

PAMPs are small conserved molecule motifs presented by a class of microbes. A well-characterized example of extracellular PAMP is the N-terminal domain of bacterial flagellin (flg22) produced by the Gram-negative bacterial pathogen *Pseudomonas syringae*. Flg22 can be recognized by *Arabidopsis thaliana* through a PRR known as FLAGELLIN-SENSING 2 (FLS2) (Chinchilla et al, 2006; Gomez-Gomez & Boller, 2000). Different from *Arabidopsis*, rice only recognizes the full-length flagellin, but tomato is able to sense a shorter epitope of flagellin (flg15)

(Meindl et al, 2000; Takai et al, 2007). Pre-treatment of *Arabidopsis* plants with flg22 induces the expression of defense-related genes and enables the plants to be more resistant to pathogenic bacteria (Zipfel et al, 2004). Besides, the *fls2* mutant *Arabidopsis* plants display enhanced susceptibility when the virulent bacteria are sprayed onto the leave surfaces. Another example of PAMP recognition is the perception of bacterial elongation factor Tu (EF-Tu) by the EF-Tu Receptor (EFR) in *Arabidopsis* (Zipfel et al, 2006). Because of the restriction that the EF-Tu perception only occurs in Brassicaceae, Lacombe *et al.* provided a successful example to engineer broad-spectrum plant resistance against important bacterial pathogens (Lacombe et al, 2010). The heterologous expression of *Arabidopsis* EFR in solanaceous plants enables their perception of EF-Tu and makes these plants more resistance to bacterial infection (Lacombe et al, 2010).

Besides flg22 and EF-Tu, there are other bacterial PAMPs, including peptidoglycans (PGNs), cold shock proteins (CSP), harpins, and lipopolysaccharide (LPS) that can also activate defense responses in plants (Gust et al, 2007; Nurnberger et al, 2004; Zipfel & Felix, 2005). Chitin, β-glucan and ergosterol are broadly recognized fungal PAMPs that are derived from cell-wall components (Nurnberger et al, 2004; Schwessinger & Zipfel, 2008). The cellulose-binding domain of *Phytophthora* lectin protein is another example of PAMPs that are found in oomycetes (Gaulin et al, 2006). Heptaglucoside, elicitins, transglutaminase are other PAMPs of oomycetes (Nurnberger et al, 2004).

PTI plays essential role in plant resistance to pathogen infection and contributes largely to the general health of plants. The intracellular signaling events involved in PTI include the activation of mitogen-activated protein kinase (MAPK) signaling as well as the calcium-dependant protein kinases (CDPKs) signaling (Block & Alfano, 2011; Schwessinger & Zipfel, 2008). These will further lead to the production of reactive oxygen species (ROS) by NADPH oxidases, the induction of ion fluxes across the cell membrane, changes in immunity-related gene expression, the activation of cell wall defenses exemplified by callose deposition, the accumulation of defense-related hormones such as salicylic acid (SA), stomata closure, and the initiation of systemic acquired resistance (SAR) (Asai et al, 2002; Block & Alfano, 2011; Luna et al, 2011; Melotto et al, 2006; Mishina & Zeier, 2007; Mittal et al, 2006; Nurnberger et al, 2004; Nurnberger et al, 1994; Ranf et al, 2011).

An elegant work reported by Melotto et al. shows that the treatments of leave epidermal peels with bacterial suspension, flg22 or LPS trigger extensive closure of stomata (Melotto et al, 2006). This PAMP-triggered stomata closure is an important mechanism of plant innate immunity at the beginning stage of bacterial infection and provides the first layer of defense. However, successful bacterial pathogens are able to re-open the stomata and proceed with infection. The best example of bacterial virulence factors that can re-open stomata is a bacterial phytotoxin called coronatine (Melotto et al, 2006). The *cor* mutants which are defective in coronatine production display reduced virulence compared with wild type bacteria when they are spray

inoculated onto the plant surface (Melotto et al, 2006). The virulence function of coronatine will be further discussed.

The Type III secretion system (T3SS)

The type III secretion system (T3SS) is a critical virulence factor of many pathogenic Gram-negative bacteria that infect plants and animals. T3SS was first discovered in 1990s (Cornelis & Van Gijsegem, 2000) and till now has been found in the majority of bacterial pathogens such as *Pseudomonas*, *Xanthomonas*, *Ralstonia*, *Erwinia*, and *Pantoea*. Different from the bacterial exotoxins, which destroys the host cells at a distance away from the site of colonization, T3SS provides a direct contact between bacteria and host cells. T3SS apparatus is highly conserved among both plant and animal pathogens and it possesses a so called "basal body" consisting of two series of rings anchored in bacterial outer membrane (outer membrane rings) as well as cell membrane (inner membrane rings) (Blocker et al, 2001; Kubori et al, 1998). A long rod-shaped "needle structure", which is anchored in the inner ring structure links the two sets of rings and protrudes from the outer ring structure to the outside environment. For an operating T3SS, a complex of proteins at the tip of this "needle structure" contribute to create a pore into the target host cell membrane and form a channel connecting the bacterial cytoplasm and the host cytosol (Marlovits et al, 2004; Mueller et al, 2008). The structure of this apparatus was nicely illustrated by Troisfontaines and Cornelis in their review (Figure 1) (Troisfontaines & Cornelis,

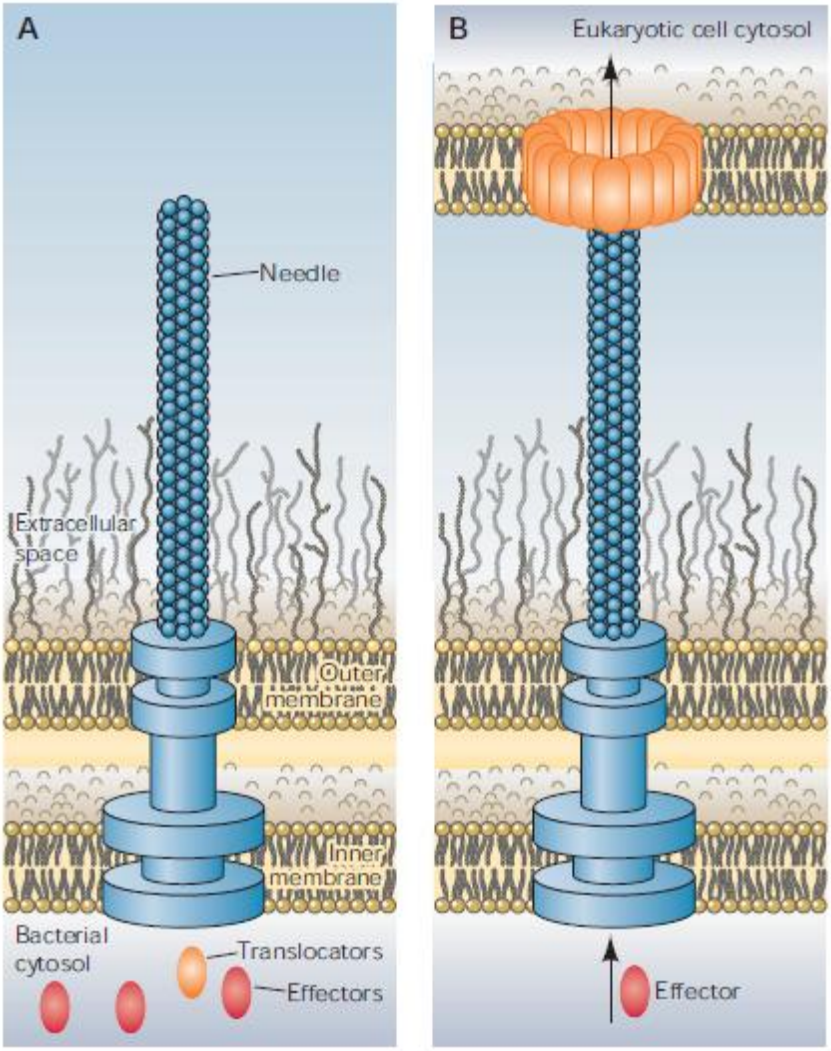
2005). In plant pathogens, the T3SS is encoded by hypersensitive response and pathogenicity (*hrp*) genes which have been mainly identified in large clusters (Bogdanove et al, 1996). A subset of *hrp* genes that are broadly conserved among different bacteria are designated as hypersensitive response and conserved (*hrc*) genes and they are largely involved in directing the secretion of T3SS substrates called the Type III-secreted effector (T3SEs) into the host cells (Alfano & Collmer, 2004).

The regulation of T3SS mainly takes place at the transcriptional level, in response to external stimuli such as pH, nutrients, temperature and divalent captions (Galan & Collmer, 1999; Hueck, 1998). After secretion, these T3SEs directly attack host targets in order to facilitate pathogen colonization of the hosts (usually in the intercellular spaces or apoplast) and develop disease symptoms (Alfano & Collmer, 2004). The significance of T3SS in bacterial pathogenesis is demonstrated by the evidence that the ability of *Pseudomonas syringae* pv. *tomato* strain DC3000 (*Pto*DC3000) to infect *Arabidopsis* plants strongly depends on its T3SS since the *Pto*DC3000 *hrp* mutants (*hrpS* or *hrcC*) cannot infect *Arabidopsis* (Hauck et al, 2003; Roine et al, 1997).

Figure 1. Structure of T3SS (Troisfontaines & Cornelis, 2005).

(A) A resting T3SS consists of two series of rings spanning the bacterial inner membrane and outer membrane and the needle protruding outside the bacterium.

(B) An operating T3SS. The translocators at the tip of the needle form a pore into the target cell membrane, and the effectors are transported into the cytosol of the target cell.



Type III-secreted effectors (T3SEs)

Effectors are defined as pathogen proteins that modulate the host cell structure and function. The best studied examples of pathogen effectors are the type III-secreted effectors produced by bacterial pathogens. Besides bacteria, filamentous eukaryotic pathogens, including oomycetes, fungi, insects, and nematodes also depend on secreted effectors to successfully infect plants and cause diseases (Kamoun, 2007).

A fundamental function of T3SEs is to inhibit PTI. A battery of T3SEs have been shown to be able to manipulate a variety of cellular processes in eukaryotic hosts for the benefit of pathogen infection (Boller & He, 2009). T3SEs-induced suppression of plant defense is termed *effector-triggered susceptibility* (ETS) (Jones & Dangl, 2006). The virulence functions of T3SEs are best-studied in the plant pathogen *P. syringae*. In the model strain *Pto*DC3000, more than 20 effectors have been so far shown to suppress PTI-associated processes (Cunnac et al, 2009). For example, AvrPto and AvrPtoB target the PAMP perception to promote bacterial infection (Xiang et al, 2011; Xiang et al, 2008). The different strategies employed by *P. syringae* effectors to facilitate bacterial pathogenesis will be further discussed.

On the other hand, plants and pathogens are always involved in an endless arms race. As a counter-attack strategy, plants have evolved nucleotide-binding leucine-rich repeat (NB-LRR) proteins to recognize specific T3SE(s), directly or indirectly, and elicit *effector-triggered immunity* (ETI) (Jones & Dangl, 2006). ETI, which often associates with localized programmed cell death at the infection sites (Boller & He,

2009; Chisholm et al, 2006; Jones & Dangl, 2006), is a robust defense response that effectively restricts the spread of the pathogen in plants. The T3SEs that are recognized by the resistant plants are designated Avirulence (Avr) proteins.

The bacterial pathogen *Pseudomonas syringae*

P. syringae has been used a model organism of bacterial pathogens infecting plants. It is a rod-shaped, Gram-negative bacterium. Exists as more than 50 different pathovars, it can infect a broad range of plant species and cause various plant diseases. For example, bacterial canker of plum caused by *P. syringae* pv. *syringae* resulted in 30% mortality rate in Germany (Hinrichs‐Berger, 2004). *P. syringae* pv. *tomato*, which causes bacterial speck of tomato, usually leads to severe production damages in wet weather (Wilson et al, 2002). The optimal temperature for *P. syringae* to cause diseases is around 12-25°C and the diseases are usually spread between plants via rain splash (Hirano & Upper, 1990).

Around 60 T3SEs have been identified in *P. syringae* and they contribute significantly to the pathogenesis via their functions in suppressing plant defense (Boller & He, 2009). Besides T3SEs, toxins produced by *P. syringae* including coronatine, syringomycin, syringopeptin, and phaseolotoxin, are also important virulence factors. These toxins modulate plant defense-related hormone pathways, associate with bacterial attachment, or target host metabolism (Bender et al, 1999; Melotto et al, 2006).

One of the most well-known *P. syringae* toxins is coronatine. A few *P. syringae* strains, such as *P. syringae* pv. *tomato*, *P. syringae* pv. *glycinea*, *P. syringae* pv. *maculicola*, produce coronatine. Early studies showed that coronatine facilitated *P. syringae* infection and promoted bacterial chlorosis in plants (Mittal & Davis, 1995). Recent experiments nicely demonstrated that coronatine structurally mimics the active form of jasmonate (JA) and effectively activates the JA signaling pathway (Feys et al, 1994; Katsir et al, 2008; Uppalapati et al, 2005). SA and JA/ethylene (ET) are two major plant hormone pathways that regulate defense responses. SA-dependent defense plays a major role in plant immunity against biotrophic and hemibiotrophic pathogens, such as the oomycete pathogen *Hyaloperonospora arabidopsidis* and *P. syringae*; whereas the JA/ET pathway promotes plant resistance against necrotrophic pathogens, such as *Botrytis cinerea* (Robert-Seilaniantz et al, 2007; Zheng et al, 2012). SA and JA/ET pathways are usually antagonistic to each other although synergism occurs under certain conditions (Mur et al, 2006). It is likely that the activation of JA signaling by coronatine leads to the suppression of SA pathway. Indeed, it has been well documented that coronatine inhibits the SA-dependant defense responses in *Arabidopsis*, such as stomata closure (Melotto et al, 2006). As a result, in wild type Columbia-0 (Col-0) plants, *Pto*DC3000 grows to a much higher level compared to the mutant *Pto*DC3118, which is defective in the production of coronatine (Melotto et al, 2006). However, in the SA biosynthetic mutant *sid-2*, *Pto*DC3000 multiplies to an equivalent level as *Pto*DC3118 (Block et al, 2005; Brooks et al, 2005). Recently,

Zheng et al. reported that coronatine activates the expression of three homologous NAC transcription factor (TF) genes. These NAC TFs are essential for the virulence function of coronatine via inhibiting SA accumulation (Attard et al, 2012; Zheng et al, 2012).

In addition, *P. syringae* is also known for its ice nucleation activity (INA) (Maki et al, 1974). Because of the presence of unique protein complex in the outer membrane, *P. syringae* can arrange water molecules into ice embryos at relative warm temperatures (Attard et al, 2012; Gurian-Sherman & Lindow, 1993). INA of *P. syringae* causes severe surface frost damage in plants to facilitate bacterial nutrient acquisition (Anderson & Ashworth, 1985; Anderson et al, 1982; Lindow, 1983). As a result, INA is another critical virulence factor of *P. syringae*. Other than its importance in bacterial pathogenesis, recent evidence suggested that this freezing role of *P. syringae* is also important for the producing of rain and snowfall in the environment (Amato et al, 2007; Attard et al, 2012; Morris et al, 2013). Besides, the INA of *P*. *syringae* has been also well applied in the food industry and in snow making (Attard et al, 2012; Nemecek-Marshall et al, 1993).

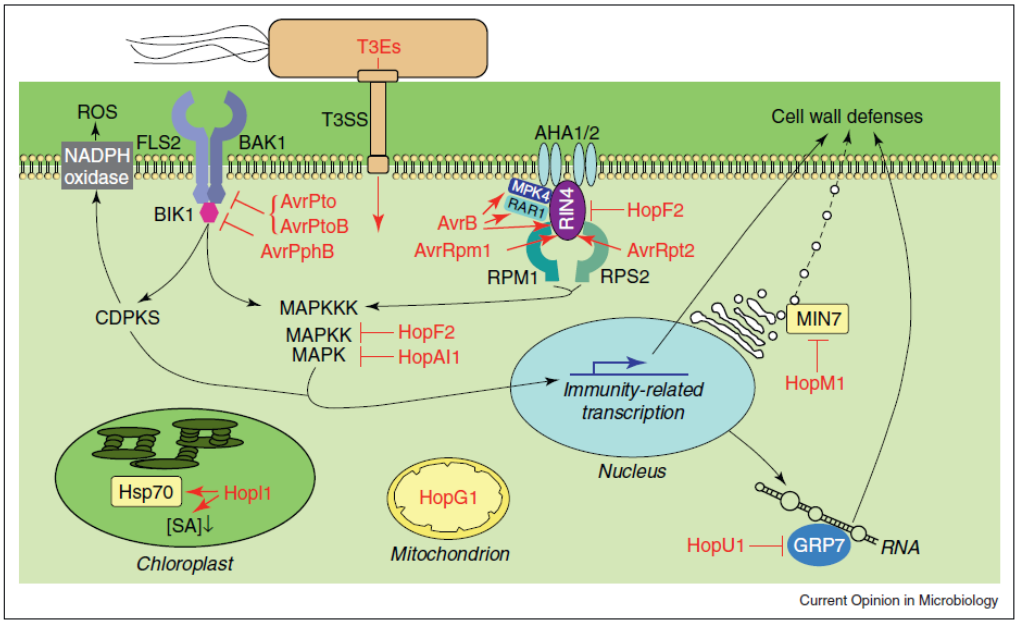
P. syringae has been well-studied for decades. The ecology of *P. syringae* has been investigated in depth, the genome sequences of multiple pathovars have been published, and the evolution of *P. syringae* has been well-characterized (Morris et al, 2013). As it infects the model plant *Arabidopsis thialian*, it is also applied as an important model system to study the molecular basis of plant-microbe interactions.

Virulence targets of *P. syringae* T3SEs

In the past 10-15 years, extensive research has been conducted to understand the molecular mechanisms underlying effector functions. In particular, the direct virulence targets of a few T3SEs in plants have been characterized. These researches suggest that many T3SEs suppress PTI and/or ETI by targeting important components in different steps of plant immunity (Galan, 2009; Grant et al, 2006). A recent review by Block and Alfano nicely summarized most known virulence targets of *P.syringae* T3SEs and how these T3SEs subvert host immunity. A figure from their review is adopted here to illustrate the concept of activation and suppression of defense responses (Figure 2) (Block & Alfano, 2011). The specific virulence targets of different effectors and the involved detailed molecular mechanisms will be further elaborated.

Figure 2. The schematic overview of the activation and suppression of plant defense (Block & Alfano, 2011).

T3SEs target different steps of plant defense for virulence functions. AvrPto and AvrPtoB target the PAMP PRRs. AvrPphB cleaves the PAMP RLK-associated kinase BIK1. HopF2 and HopAI1 modify kinases in the MAPK cascade. HopM1 induces the degradation of MIN7 to interfere with vesicle trafficking. HopU1 ADP-ribosylates RNA-binding proteins. HopI1 activates Hsp70 and suppresses SA accumulation. AvrRpt2, AvrRpm1, and AvrB cleave or modify RIN4 that regulates PTI. AvrB also interacts with RAR1 as well as MAP kinase 4 (MPK4) that form a complex with RIN4.



Effectors that target PRRs perception or MAPK signaling

AvrPto and AvrPtoB are two sequence-distinct effectors secreted by *Pto*DC3000 that block the function of the PRR complex.

Xiang et al. reported that AvrPto physically interacts with and inhibits the kinase activity of FLS2 and EFR, and this binding activity is responsible for the virulence function of AvrPto (Xiang et al, 2008). BRI1-ASSOCIATED KINASE 1 (BAK1), an *Arabidopsis* receptor-like kinase (RLK), was reported to bind to both AvrPto and AvrPtoB (Shan et al, 2008). As BAK1 is a co-receptor of FLS2 and EFR, it plays a critical role in flagellin- or EF-Tu- induced defense. The associations of AvrPto or AvrPtoB with BAK1 disrupt the formation of FLS2/EFR-BAK1 complex and diminish PAMP perception. However, a recent research by Xiang *et al*. showed conflicting result that FLS2 but not BAK1 is targeted by AvrPto in planta (Xiang et al, 2011). As a result, more experiments are required to reveal the true virulence targets of these effectors.

Besides, the C-terminal domain of AvrPtoB, which is distinguished from the FLS2/BAK1-associating N-termianl domain, possesses an E3 ubiquitin ligase activity, which catalyzes the ubiquitination, and the subsequent degradation of FLS2 by 26S proteasome. (Gohre et al, 2008). This E3 ubiquitin ligase activity of AvrPtoB is required for its ability to enhance the virulence function of *Pto*DC3000 (Gohre et al, 2008). The C-terminal E3 ligase domain of AvrPtoB can also promote the ubiquitination and degradation of another LysM receptor kinase CHITIN ELICITOR

RECEPTOR KINASE 1 (CERK1) in *Arabidopsis* (Gimenez-Ibanez et al, 2009), as well as a tomato kinase Fen, which is involved in an unique ancient ETI pathway (Rosebrock et al, 2007).

The interference of AvrPto and AvrPtoB with the PAMP perception can nicely explain why these effectors are able to suppress a variety of PTI-associated signaling events and defense responses including the activation of MAPK signaling cascade, the expression of MAMP-specific early-defense genes and defense-related small RNAs, and the induction of callose depositon (Hauck et al, 2003; Mittal et al, 2006; Navarro et al, 2008).

Another *P. syringae* T3SE AvrPphB was reported to be a papain-like cysteine protease that cleaves the *Arabidopsis* protein kinase PBS1 (Shao et al, 2003). PBS1 forms a complex with an R protein RPS5 in the absence of AvrPphB. The cleavage of PBS1 by AvrPphB results in the conformational changes of RPS5 and initiates ETI (Ade et al, 2007). However, recent studies showed that in the plants lacking RPS5, AvrPphB significantly suppressed PTI responses, which was illustrated by the reduced oxidative burst and callose deposition in the *AvrPphB* transgenic *Arabidopsis* (Zhang et al, 2010). Except for PBS1, AvrPphB proteolytically cleaves additional PBS1-like (PBL) kinases including BIK1, PBL1, and PBL2 in order to inhibit PTI. As a RLK-associating kinase, BIK1 is critical for defense responses triggered by three PAMPs (flg22, elf18 and chitin). Unlike the loss of function *bik1* mutants that showed significantly compromised PTI response to several PAMPs, *Arabidopsis* plants

lacking *PBS1* only displayed minimal reduction in PTI response. Besides, the T-DNA insertion mutants of PBL1 and PBL2 selectively exhibited diminished defense responses to certain PAMPs (Zhang et al, 2010). These indicated that PBS1, PBL1, and PBL2, to a less extent, are required for the PTI. It was proposed that PBS1 was evolved to detect AvrPphB by mimicking its virulence substrates, like BIK1, in plants (Ade et al, 2007; Warren et al, 1999).

HopAI1, another effector protein, also targets early steps of PAMP-induced signaling pathway (Zhang et al, 2007). Functions as a phosphothreonine lyase, HopAI1 dephosphorylates MPK3 and MPK6 which play important roles in plant defense. The inhibition of MAPK cascade by HopAI1 diminishes the activation of PAMP response genes and callose deposition. Interestingly, OspF, a T3SE secreted by all the four known pathogenetic species of *Shigella* (a genus of mammal pathogen), was also shown to dephosphorylate MAPKs in animals (Li et al, 2007). This suggests that HopAI1 and OspF belong to an evolutionarily conserved family of T3SEs produced by both plant and animal pathogens to overcome host defense using a conserved mechanism.

Another effector that can target MAPK signaling for virulence is HopF2 secreted by *Pto*DC3000. HopF2 directly targets *Arabidopsis* MAP KINASE KINASE5 (MKK5) and blocks the kinase function of MKK5 via its activity as a mono-ADP-ribosyltransferase, and as a result inhibits PTI (Wang et al, 2010).

Effectors that targets other defense-related processes

Besides targeting the PAMP perception or MAPK cascade, effectors also impact the processes downstream of PRR signaling or other consequent defense related events. HopM1 is another effector that helps *P. syringae* to successfully subvert host immunity by promoting the turnover of an immunity-associated protein called AtMIN7 in *Arabidopsis* (Nomura et al, 2006; Nomura et al, 2011). Confocal examination revealed that HopM1 and AtMIN7 co-located to *trans*-Golgi network /early endosome (Nomura et al, 2011). HopM1 interacts with AtMIN7 through its N-terminus and promotes the ubiquitination and degradation of AtMIN7 via the 26S proteasome pathway. As AtMIN7 belongs to adenosine diphosphate ribosylation factor-guanine nucleotide exchange factor (ARF-GEF) protein family, members of which are key components involved in plant vesicle transport, the degradation of AtMIN7 by HopM1 significantly disrupts the *Arabidopsis* vesicle trafficking pathway, which is required for exporting defense compounds to the cell wall or apoplastic region (Collins et al, 2003; Nomura et al, 2006). However, the sequence analysis of HopM1 revealed that this effector does not have any similarity to known components that involved in ubiquitination/proteasome pathways; therefore, the detailed mechanisms of how HopM1 brings AtMIN7 to proteasome system are still unclear.

Other than HopF2, HopU1 is another mono-ADP-ribosyltransferase secreted by *Pto*DC3000. It suppresses plant defense by modifying glycine-rich RNA-binding protein 7 (GRP7) and inhibiting its RNA binding activity (Fu et al, 2007). The

Arabidopsis plants lacking *grp7* shows increased susceptibility to *P. syringae* infection (Fu et al, 2007), suggesting that disabling post-transcriptional regulations of specific mRNAs might be important for defeating plant innate immunity. However, the immunity-related RNAs that HopU1 interferes with remained unclear until recently Nicaise *et al*. reported that HopU1 inhibits the interaction between GRP7 and the transcripts of *fls2* and *efr* via ribosylating the RNA binding domain of GRP7 (Nicaise et al, 2013). This inhibition is correlated with the observation that the FLS2 protein level is suppressed in plants infected by *P. syringae* in a HopU1-dependant manner (Nicaise et al, 2013). HopU1 applies a very unique strategy to subvert host immunity.

HopI1 also has known virulence target(s) in plants. HopI1 specifically localizes in the plant cell chloroplasts, where the important plant defense hormone SA is synthesized (Jelenska et al, 2007). Over-expression of HopI1 in *Arabidopsis* causes the suppression of SA accumulation and the inhibition of SA-dependant defense responses (Jelenska et al, 2007). The potential J domain of HopI1 (Guttman et al, 2002; Kelley, 1998) is important for this effector to interact with and stimulate the ATPase hydrolysis activity of the plant heat shock protein Hsp70 in vitro (Jelenska et al, 2010). Hsp70 is necessary for the virulence function of HopI1 because the growth difference between *P. syringae* pv. *maculicola* strain ES4326 (*Pma*ES*4326*) and its mutant *Pma*Δ*hopI1*, which does not produce HopI1, is highly reduced in both the *Arabidopsis* and the *N. benthamiana* plants lacking *Hsp70-1*, when compared to that

in the wild type plants (Jelenska et al, 2010). However, how does the Hsp70 activity correlate with SA production is still poorly understood.

Effectors that target PTI or ETI regulators

AvrRpt2 is the first T3SE with its direct plant target(s) identified and characterized. AvrRpt2 functions as a cysteine protease in plants with a host "helper" cyclophilin (Axtell et al, 2003; Coaker et al, 2005). In *Arabidopsis*, AvrRpt2 cleaves an RPM1-interacting protein 4 (RIN4), a negative regulator of PTI (Kim et al, 2005). RIN4 was also reported to interact with plasma membrane (PM) H+-ATPases to modulate stomatal apertures during pathogen infection (Liu et al, 2009). The *rin4* knock-out *Arabidopsis* lines show reduced PM H+-ATPases activity and the inhibition of stomata re-opening by virulent *Pto*DC3000 which produces coronatine. The disappearance of RIN4 as a result of AvrRpt2 cleavage is guarded by an R protein called RPS2, which initiates AvrRpt2-triggered immunity, an ETI response (Axtell & Staskawicz, 2003). AvrRpt2 facilitated the growth of a T3SS deficient bacterial mutant strain *Pto*DC3000 *hrcC* and inhibited PAMP-induced defense responses like callose deposition and pathogenesis-related (PR) gene expression (Kim et al, 2005). As RIN4 also interacts with at least two other effectors AvrRpm1 and AvrB as well as two R proteins, the cleavage of RIN4 may impact the stability of this complex and benefit the bacterial infection. However, the exact significance of RIN4 elimination to the virulence function of AvrRpt2 is still unclear. Moreover, it was shown that the

virulence function of AvrRpt2 was also detected in *Arabidopsis* plants lacking *RIN4* (Lim & Kunkel, 2004), suggesting that the virulence activity of AvrRpt2 may rely on other host targets, in addition to RIN4.

As mentioned above, two unrelated effectors, AvrRpm1 and AvrB were also found to interact with RIN4 and their interactions result in the phosphorylation of RIN4 (Mackey et al, 2002). The modified RIN4 as a "Guardee" is required for the recognition of AvrRpm1 and AvrB by an R protein RPM1, which result in the subsequent HR response. Similar to AvrRpt2, both AvrRpm1 and AvrB maintain their virulence activities in the absence of *rin4* and *rpm1* (Belkhadir et al, 2004), suggesting that RIN4 might be a decoy of the true virulence target(s) of these T3SEs (van der Hoorn & Kamoun, 2008). In addition, RIN4 was recently found to be targeted by HopF2. The modification (ADP-ribosylation) of RIN4 by HopF2 may interfere with the interaction between AvrRpt2 and RIN4, thereby preventing AvrRpt2-induced ETI by blocking the cleavage of RIN4. (Wang et al, 2010; Wilton et al, 2010).

Besides RIN4, AvrB was also found to interact with RAR1 in vivo (Shang et al, 2006). RAR1 is a cochaperone of HSP90 and both of them play important roles in stabilizing several R proteins (Azevedo et al, 2006; Bieri et al, 2004; Tornero et al, 2002). In addition to its essential role in ETI, RAR1 also negatively regulates PTI, which is shown by the evidence that after flg22 treatment, wild type plants have fewer callose deposits than *rar1-20* mutant plants (Shang et al, 2006). AvrB enhances the

susceptibility of *Arabidopsis* plants that lacks *rpm1* to a non-pathogenic bacterium strain *Pto*DC3000 *hrpL*, in a RAR1-dependant manner. Meanwhile, RAR1 is also required for AvrB to suppress the PAMP-induced cell wall defense (Shang et al, 2006). In addition, it was also recently reported that AvrB interacted with and phosphorylated MPK4 (Cui et al, 2010), which negatively regulates plant defense against *P. syringae*. Their results suggested that RAR1, HSP90, MPK4, and RIN4 constitute a pathway directly targeted by AvrB to facilitate the pathogenesis of *P. syringae*.

YopJ family of T3SEs

The YopJ family of T3SE is one of the most widely distributed bacterial effector families produced by both animal and plant pathogens. The founding member of this family is YopJ of *Yersinia pestis*, the causal agent of black death (Plague) (Hinnebusch, 2005). Several homologues of YopJ were also discovered from other animal pathogens like *Vibrio* (VopA and VopJ), *Salmonella* (AvrA) and *Aeromonas* (AopP) (Ma et al, 2006). YopJ-like effectors are essential virulence factors of these animal pathogens that cause life-threatening diseases of hosts. YopJ-like effectors are also produced by numerous plant pathogens (Ma et al, 2006), such as *Xanthomonas* (AvrXv4, AvrRxv, AvrBsT and XopJ), *P. syringae* (HopZ1, HopZ2 and HopZ3), *Ralstonia* (PopP1 and Pop2), and *Erwinia* (ORFB). These effector proteins play important roles in disrupting plant host immunity during bacterial infection. A YopJ homologue Y4LO was found in plant microsymbiont *Rhizobium* sp. strain NGR234

(Ma et al, 2006; Yang et al, 2009). 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 (Barrett & Rawlings, 2001). This catalytic core is required for both virulence and avirulence functionalities of YopJ family effectors (Lewis et al, 2011; Zhou et al, 2009).

Enzymatic activity of YopJ

YopJ inhibits host inflammatory responses and promotes apoptosis of immune cells (Orth et al, 1999). Inside the host cytosol, YopJ blocks MAPK and NFκB signaling pathways by directly targeting MAP kinase kinases (MKKs) and the NF-κB inducing kinase (NIK), IKKα/β complex (Ορτη ετ αλ, 1999). Although YopJ was initially characterized as a cysteine protease (Orth et al, 2000), Mukherjee *et al.* reported that YopJ acetylated MKK6 on the Ser^{207} and Thr^{211} residues, which are located in the activation loop of the kinase (Mukherjee et al, 2006). This study demonstrated that YopJ acts as an acetyltransferase in animals. Furthermore, a host co-factor inositol hexakisphosphate (IP6) is required for the full acetyltransferase activity of YopJ (Mittal et al, 2010). Since the serine and threonine residues are conserved throughout the MKK super family, it is suggestive that other MKKs may also be acetylation targets of YopJ. Indeed, YopJ acetylates two serine residues in the activation loop of MKK1/2, a threonine residue in the activation loop of the IKKα/β

kinases (Mittal et al, 2006), and key serine/threonine residues in the activation loop of TGFβ-activated kinase 1 (TAK1) (Paquette et al, 2012). As such, YopJ-mediated acetylation on these key catalytic residues inhibits the activation of MKKs, IKKα/β and TAK1. This is verified by in vitro assays demonstrating that MKK6, IKKβ or TAK1were no longer phosphorylated by upstream kinases in the presence of YopJ (Mukherjee et al, 2006; Paquette et al, 2012), which prevents them from propagating defense signaling through phosphorylating the downstream molecules.

Enzymatic activities of other effectors from YopJ superfamily

Several other members of YopJ effector family have also been reported to have acetyltransferase activities. Similar to YopJ, VopA modifies its target proteins MKKs in animal hosts and the acetylation of these host targets blocks their phosphorylation and the subsequent defense signal transduction (Trosky et al, 2007). Different from YopJ, VopA not only acetylates the conserved serine and threonine residues but also a conserved lysine residue in the catalytic loop of MKKs (Trosky et al, 2007). In addition, unlike YopJ, VopA does not block the NFκB pathway. Another YopJ-like effector AvrA from *Salmonella typhimurium* also acetylates MKKs (Jones et al, 2008).

PopP2 produced by the plant pathogen *Ralstonia solanacerum* possesses autoacetylation activity, which is essential for its recognition in resistant plants. However, whether PopP2 can modify its target proteins in the host remains unknown (Tasset et al, 2010). Recent studies showed that in the presence of IP6, *P. syringae*

YopJ-like effector HopZ1a acetylated tubulin in vitro. Modification of tubulin is associated with dissembling the microtubule cytoskeleton, which may contribute to bacterial infection (Lee et al, 2012).

Other YopJ-like T3SEs produced by plant pathogens appear to have various enzymatic activities. AvrXv4 of *Xanthomonas campestris* was reported to be a small ubiquitin-related modifier (SUMO) protease (Roden et al, 2004). AvrBsT, also from *Xanthomonas*, exhibits a weak cysteine protease activity in vitro (Szczesny et al, 2010). The same weak cysteine protease activities were also reported for HopZ1, HopZ2 and HopZ3 in vitro (Ma et al, 2006).

HopZ1 effector family in *P. syringae*

Three YopJ homologs, designated HopZ1, HopZ2 and HopZ3, were identified from various *P. syringae* strains (Ma et al, 2006). Evolutionary and phylogenetic analyses indicate that HopZ1 is an ancestral virulence factor in *P. syringae* that was under strong positive selections (Ma et al, 2006). HopZ1 group contains at least three functional allelic variants (HopZ1a, HopZ1b and HopZ1c) and two degenerate alleles, with HopZ1a resembling the most ancestral allele form (Ma et al, 2006). HopZ1b is produced by *P. syringae* pv. glycinea (*Pgy*) strains, which are the causal agents of bacterial blight disease on soybean (*Glycine max*) (Ma et al, 2006). Although HopZ1b shares approximately 81% similarity in the full-length amino acid sequence with HopZ1a, they triggered distinct plant responses. HopZ1b$_{Pgy\text{BR1}}$ (HopZ1b in *Pgy* strain

BR1; hereafter HopZ1b) promotes *P. syringae* infection in soybean; whereas the HopZ1a$_{PsyA2}$ (HopZ1a in *P. syringae* pv. *syringae* strain A2; hereafter HopZ1a) triggers an HR in soybean cultivar Williams 82 and *Arabidopsis thaliana* accession Col-0 (Zhou et al, 2009). Because HopZ1c is a shorter allelic variant produced by *P. syringae* pv. *maculicola* ES4326 with unknown activity or function so far (Lewis et al, 2008; Zhou et al, 2009), most research has been done on HopZ1a and HopZ1b.

Avirulence and virulence functions of HopZ1 in Arabidopsis thaliana

In Arbidopsis, HopZ1a causes strong hypersensitive response in the ecotype Col-0 (Ma et al, 2006). By using a reverse genetic screen, an *Arabidopsis* R protein HOPZ-ACTIVATED RESISTANCE 1 (ZAR1) was found to be required for the recognition of HopZ1a (Lewis et al, 2010). During bacterial infection, HopZ1a exhibits evident virulence function in *zar1-1* mutant *Arabidopsis* plants, which is abrogated in HopZ1a recognition. Importantly, the recognition of HopZ1a by ZAR1 depends on the catalytic cysteine residue (Cys^{216}) and a potential myristoylation site (Gly^{2}) in the N-terminus of HopZ1a (Lewis et al, 2010; Ma et al, 2006). This indicates that HopZ1a-triggered defense response requires its enzymatic activity as well as the plasma membrane localization. Unlike HopZ1a, HopZ1b only caused HR in about 1/4 of the *Arabidopsis* leaves and this recognition is independent of ZAR1 (Lewis et al, 2008; Lewis et al, 2010).

Macho *et al.* discovered that HopZ1a-triggered plant immunity is additive to the defense responses triggered by AvrRpt2, AvrRps4 and AvrRpm1 as shown by competitive index assays (Macho et al, 2010). Besides, HopZ1a also suppresses systemic acquired resistance (SAR) triggered by other bacterial pathogens (Macho et al, 2010). Detailed analyses demonstrated that HopZ1a-induced plant defense is independent of known defense-related hormone pathways like SA, JA, and ET-dependant resistance.

Recently, *Arabidopsis* tubulin and polymerized microtubules were reported to be virulence targets of HopZ1a (Lee et al, 2012). Through the interaction and modification with tubulin, HopZ1a damaged *Arabidopsis* microtubule networks, thus contributing to *P. syringae* multiplication (Lee et al, 2012). The catalytic cysteine residue is also required for this virulence function of HopZ1a. However, the acetylation of tubulin by HopZ1a is rather weak and how the aceylation of tubulin interferes with the destruction of microtubule networks is unclear.

Avirulence and virulence functions of HopZ1 in soybean

In soybean, HopZ1a triggers strong defense responses, whereas HopZ1b promotes the multiplication of non-host pathogens such as *P. syringae* pv. *phaseolicola* 1302A (*Pph*1302A) (Ma et al, 2006; Zhou et al, 2009). The catalytic cysteine residue is required for the HR triggered by HopZ1a and the infection promoting activity of HopZ1b. In addition, HopZ1a(G2A) mutant completely abolishes the HR-triggering

ability of HopZ1a, whereas HopZ1b(G2A) mutant still benefits the growth of *Pph*1302A similar to the wild type HopZ1b (Zhou et al, 2009). These results suggest that the potential myristoylation site is only involved in host recognition of HopZ1a but not required for the virulence function of HopZ1b in soybean.

As HopZ1a and HopZ1b share very high sequence identity, regions and residues that determine the HopZ1 allelic specificity were determined. Domain shuffling experiments showed that the central domains of HopZ1 ($HopZ1a_{63-203}$ and the $HopZ1b_{59-199}$) are responsible for the allelic specificity (Morgan et al, 2010). Further mutagenesis experiments revealed that the replacement of Cys^{141} in HopZ1a with Lys^{137} at corresponding site in HopZ1b abolished the HR-eliciting activity of HopZ1a (Morgan et al, 2010). Phylogenetic analysis supports the notion that HopZ1a resembles the ancestral allelic form, from which HopZ1b was evolved in soybean pathogens to evade host recognition (Ma et al, 2006). Therefore, the substitution of C141 with K137 in HopZ1b might be a key step in HopZ1 evolution.

Recently, efforts in our lab identified potential virulence targets of HopZ1 in soybean using yeast two-hybrid screening (Zhou et al, 2011). One of the HopZ1-interacting proteins (ZINPs), designated as ZINP1 (2-hydroxyisoflavanone dehydratase, or GmHID1), is a key enzyme in the soybean isoflavone biosynthetic pathway (Akashi et al, 2005; Faraj & Vasanthan, 2004). This links the HopZ1 activity to plant defense because isoflavones are precursors of phytolexins, which are antimicrobial compounds, in soybean (Yao et al, 2011). Our data demonstrated that

GmHID1 is a positive regulator of soybean basal defense (Zhou et al, 2011). Importantly, HopZ1 induces the degradation of GmHID1 when these two proteins are co-expressed in plant cells. Furthermore, decreased accumulation of isoflavones was observed in soybean infected with *P. syringae* producing HopZ1b, but not the catalytic mutant of HopZ1b (Zhou et al, 2011). These data lead to the conclusion that HopZ1 directly targets GmHID1 in soybean to inhibit isoflavone biosynthesis and promote bacterial infection.

Avirulence and virulence functions of HopZ1 in Nicothiana benthamiana

In *N. benthamianan*, both HopZ1a and HopZ1b trigger HR-like cell death when transiently expressed using *Agrobacterium*-mediated expression (Ma et al, 2006), and the catalytic cysteine residues are required for both of their HR eliciting ability. However, the potential N-terminal myristoylation site only enhances the development of cell death triggered by HopZ1b, but not by HopZ1a (Zhou et al, 2009). In addition, only HopZ1b-triggered cell death can be blocked by $HopZ3_{PsyB728a}$ (HopZ3 hereafter), a YopJ homolog that was found to suppress ETI elicited by AvrPto, HopAA1, HopM1, and HopAE1 in *N. benthamiana* (Vinatzer et al, 2006). It is likely that HopZ1a and HopZ1b are recognized through different pathways in *N. benthamiana*, as a result of their allelic specificities.

HopZ1a activates JA signaling

An interesting discovery by Macho *et al* is that HopZ1a suppressed the induction of *PR-1* and *PR-5*, which are two genes associated with plant defense against biotrophic pathogens or SAR (Macho et al, 2010; Ryals et al, 1996). On the contrary, the transcription level of *PDF1.2*, a marker for JA and ET defense pathway, is much higher in *Arabidopsis* plants infected with *Pto*DC3000(HopZ1a) when compared to that in plants inoculated with *Pto*DC3000 or *Pto*DC3000(AvrRpt2) (Macho et al, 2010). These results suggest that HopZ1a could activate JA signaling during bacteria-plant interactions. Indeed, we found that a jasmonate ZIM-domain (JAZ) protein interacted with HopZ1a in the yeast two-hybrid screening. As JAZs are important negative regulators in JA signaling (Chini et al, 2007), it raised our great interest to examine the hypothesis that the virulence function of HopZ1a is associated with its JA activating ability.

Jasmonates are important defense hormones in plants

Jasmonates are plant-specific oxylipin signaling molecules derived from tri-unsaturated fatty acids α-linolenic acid (18:3) (α-LeA) or its homologous hexadecatrienoic acid (16:3), found in galactolipids of chloroplast membranes (Kombrink, 2012). JA-Ile is the known active form involved in JA signaling (Katsir et al, 2008; Sheard et al, 2010). As one of the major phytohormones, JA regulates various aspects of plant growth and development processes including seed

germination and seedling development (Corbineau et al, 1988), root growth (Staswick et al, 1992), fruit ripening (Perez et al, 1997), fertility (Stintzi & Browse, 2000), anthocyanin accumulation, trichome development (Qi et al, 2011), and plant senescence (Xiao et al, 2004). Importantly, JA signaling, which plays an essential role in plant defenses against necrotrophic pathogens and herbivorous insects (Feys et al, 1994; McConn et al, 1997), is usually believed to be antagonistic to SA signaling, which is critical to plant defense against biotrophic and hemibiotrophic pathogens, although synergism occurs under certain conditions (Mur et al, 2006). In addition, JA also manipulates plant responses to several abiotic stressors such as mechanical wounding (Reymond et al, 2000) , UV radiation (Conconi et al, 1996), salt and drought (Zhu, 2002).

Jasmonate Zim-domain proteins (JAZ)

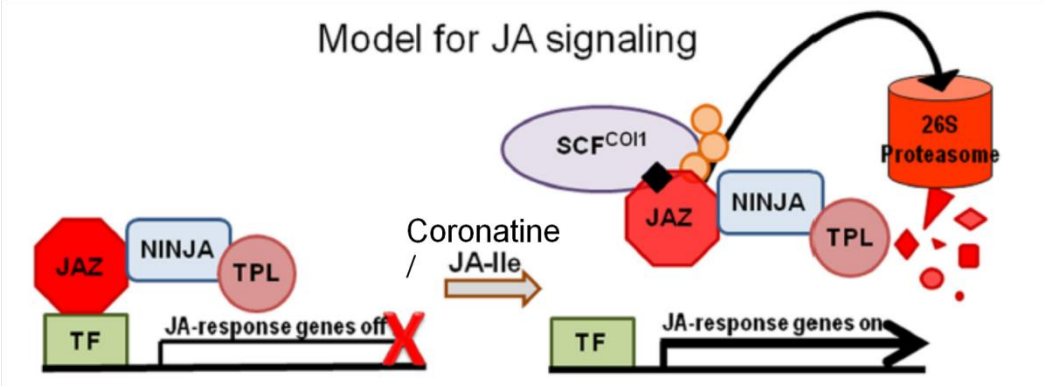
In 2007, a new family of 12 proteins was discovered in *Arabidopsis* that designated as jasmonate ZIM-domain (JAZ) proteins (Chini et al, 2007; Thines et al, 2007; Yan et al, 2007). JAZs (the *Arabidopsis* JAZ proteins are called JAZs for short in the general introduction part of this dissertation) are important components of the JA receptor complex and key transcription repressors regulating JA-responsive genes (Chini et al, 2007; Sheard et al, 2010). Furthermore, through interacting with other proteins, JAZs act as coordinators in the crosstalks between JA signaling and other

hormone pathways to impact multiple aspects of plant growth, defense and other metabolic activities (Kazan & Manners, 2012).

Under normal plant growth conditions when the JA hormone levels are low and in the absence of JA-Ile mimic molecule coronatine, JAZ proteins bind to MYC2 and other JA signaling transcription factors to inhibit the expression of JA-responsive genes. When the concentrations of JA-Ile or coronatine reach a threshold in response to wounding, insect feeding or necrotic pathogen infection, JAZ proteins are recruited to the F-box protein CORONATINVE INSENSITIVE 1 (COI1), a key component of the E3 ubiquitin ligase SCF^{COI1}. The targeting of JAZs by COI1 leads to the subsequent ubiquitination and degradation of JAZs via the 26S proteasome pathway, thereby releasing the MYC and other transcription factors and de-repressing JA signaling (Chini et al, 2007; Thines et al, 2007). A figure adopted from a review by Wager and Browse can nicely elaborate a model for the action of JA-Ile and coronatine and how JAZs regulate JA signaling (Figure 3) (Wager & Browse, 2012).

Figure 3. A model for how JA signaling in plants (Wager & Browse, 2012).

In the absence of JA-Ile or coronatine, JAZ proteins bind JA signaling transcription factors and recruit co-repressors, including NINJA and TOPLESS (TPL), to inhibit the expression of JA-responsive genes. When the concentrations of JA-Ile or coronatine reach a threshold in response to wounding, insect feeding or necrotic pathogen infection, JAZ proteins are recruited to the F-box protein COI1, a key component of the E3 ubiquitin ligase SCF^{COI1}. The targeting of JAZs by COI1 leads to the subsequent ubiquitination and degradation of JAZs via the 26S proteasome pathway, thereby releasing the transcription factors and de-repressing JA signaling.



Structure of the JAZ proteins

JAZ proteins belong to plant specific TIFY transcription factors, which are classified into four sub-families: TIFY, PEAPOD (PPD), JAZ and ZIM-like (ZML), according to their different domain architectures (Bai et al, 2011). Unlike other TIFY transcription factors that contain a GATA-Zinc finger, JAZ proteins lack a known DNA-binding domain, therefore requiring interaction with other DNA-binding proteins to function as transcription regulators (Vanholme et al, 2007).

All 12 JAZ proteins in *Arabidopsis* contain two highly conserved sequence motifs: the ZIM domain with TIF[F/Y]XG signature in the central portion of the protein (Vanholme et al, 2007), which is required for repressing JA signaling and mediating the homo- and hetero-interactions between different JAZ proteins (Chini et al, 2009; Chung & Howe, 2009; Pauwels et al, 2010). The Jas domain with the concensus sequence $SLX_2FX_2KRX_2RX_5PY$ at the C-terminus of the protein mediates the interaction of JAZ with MYC transcription factors and COI1, thereby plays a critical role in the repression of JA signaling and the stability of JAZ proteins (Chini et al, 2007; Thines et al, 2007; Yan et al, 2007). *JAZ* mutants or *Arabidopsis* transgenic plants expressing JAZ proteins lacking the Jas domain exhibit dominant insensitivity to exogenous application of JA (Chini et al, 2007; Thines et al, 2007; Yan et al, 2007). The function of the weakly conserved N-terminal region of JAZ proteins has not been well characterized (Thines et al, 2007) (Hou et al, 2010).

JAZ targets in plants

JAZ interaction with COI1

The critical role of COI1 in JA signaling was first discovered by a screen, in which *Arabidopsis* seedlings that are resistant to root growth inhibition by coronatine were characterized (Feys et al, 1994). *coi1-1* is one of the first mutants that exhibit significant JA insensitivity and deficiency in all JA-related responses (Feys et al, 1994; Xie et al, 1998). COI1 is an F-box protein and important recognition component of the SCF^{COI1} complex, a member of the Skip/Cullin/F-box (SCF) family of E3 ubiquitin ligases (Xie et al, 1998). COI is closely related to transport inhibitor response 1 (TIR1), the recognition component of E3 ubiquitin ligase SCF^{TIR1}, which targets and degrades the auxin receptor AUX/IAA proteins (Dharmasiri et al, 2005).

The conserved C-terminal Jas domain of JAZs mediates the interaction between JAZs and COI1 (Melotto et al, 2008). In particular, two critical arginine residues (Arg^{205} and Arg^{206} of JAZ1 and Arg^{223} and Arg^{224} of JAZ9) within the Jas domain were required for the interaction with COI1. *Arabidopsis* plants over-expressing a JAZ1 mutant with Arg^{205} and Arg^{206} replaced with alanines exhibit strong JA insensitivity (Melotto et al, 2008).

Recently, the COI1-JAZ complex, rather than COI1 alone, was found to function as a high affinity JA receptor (Sheard et al, 2010). The crystal structure of COI1-ASK1 (SCF^{TIR1} adaptor) complex with JA-Ile and the JAZ degron peptide showed

that JA-Ile interacts with COI1 residues in the leucine-rich-repeat/loop structure, which constitutes the ligand-binding pocket. Although most of the JA-Ile ligand is buried inside of the binding sites of COI1, the keto group of JA and the carboxyl group of Ile are exposed to allow interaction with JAZs (Sheard et al, 2010; Wager & Browse, 2012). A bipartite structure of JAZ1 is important for the binding of JAZ1 to COI1 in the presence of JA-Ile. This bipartite JAZ1 degron consists of a conserved α-helix to strengthen the interaction between JAZ1 and COI1, and a loop region to trap the hormone in its binding pocket (Sheard et al, 2010). In addition, a third important component of the JA co-receptor complex, Inositol pentakisphosphate (IP_5), was identified as a co-factor that interacts with both COI1 and JAZ (Sheard et al, 2010).

JAZs interact with basic-helix-loop-helix (bHLH) transcription factors

MYC2 is the first DNA-binding transcription factor that regulates JA responses (Lorenzo et al, 2004). Using mutant screens, several *jasmonate insensitive* (*jai*) loci were identified in *Arabidopsis* (Lorenzo et al, 2004). One of these loci, *jai/jin1* , encodes a nuclear localized basic-helix-loop-helix-leucine zipper (bHLH-zip)-type transcription factor-AtMYC2, which regulates the expressions of *VSP2*, *LOX3* and *TAT* genes that are involved in JA-mediated responses to wounding. MYC2 also represses the expression of a set of genes such as *PDF1.2*, *PR1* and *PR4*, which are involved in defense responses against pathogens. However, unlike the *coi1* mutants, *myc2* mutants are still fertile and only exhibit partial JA insensitivity phenotypes

(Fernandez-Calvo et al, 2011), suggesting that MYC2 is not the only transcription factors regulating JA responsive genes.

Chini et al. reported that MYC2 directly interacts with JAZ3 (Chini et al, 2007). In the absence of JA or coronatien, JAZ3 binds with MYC2 and inhibits its activity as transcription factor. However, JAZ proteins are degraded in the presence of JA, thus resulting in the expression activation of JA-responsive genes by MYC2. Despite the important role of MYC2 in JA signaling, the relative weak phenotype of *myc2* mutnat compared with *coi1* suggests the involvement of other transcription factors in JA-induced responses (Fernandez-Calvo et al, 2011).

In 2011, by means of yeast two-hybrid screening and tandem affinity purification, three groups of researchers independently identified two new JAZ targets, bHLH transcription factors MYC3 and MYC4, which are phylogenetically related to MYC2 (Cheng et al, 2011; Fernandez-Calvo et al, 2011; Niu et al, 2011). To compare the binding specificity of MYC3 and MYC4 with that of MYC2, Ferna´ndez-Calvo *et al.* examined the DNA binding sites of MYC3 and MYC4 by protein binding microarray assay (Fernandez-Calvo et al, 2011). They showed that the consensus DNA binding sites of MYC3 and MYC4 are quite similar to that of MYC2, which suggests that the MYC transcription factors are at least partially redundant in their functions. This is further confirmed by the evidence that *myc3* and *myc4* mutants impair plant responses to JA hormone and further enhance JA insensitivity of *myc2* mutants (Fernandez-Calvo et al, 2011).

The N-terminal JAZ interacting domain (JID) of MYC2/MYC3/MYC4 is critical for their interactions with JAZs (Fernandez-Calvo et al, 2011). JID domain is also present in other bHLH TFs, suggesting that JAZs may interact with more bHLH TFs in addition to MYC proteins. This hypothesis is supported by the result that *myc2/myc3/myc4* triple mutant renders plants as insensitive as *coi1* mutants to several but not all JA responses, such as defense against necrotrophic pathogens and insects (Fernandez-Calvo et al, 2011). Indeed, three additional bHLH TFs, Glabra3 (GL3), TRANSPARENT TESTA8 (TT8) and GLABRA3 (EGL3) also interact with JAZs (Qi et al, 2011). The JA-induced anthocyanin accumulation and trichome initiation are severely disrupted in *gl3 egl3 tt8* triple mutants when compared to wild type plants, indicating important roles of these bHLH TFs in JA-regulated plant responses (Qi et al, 2011).

JAZs interact with R2R3 MYB transcription factors

Using JAZ1 as the bait protein, another yeast two-hybrid screen showed that JAZ1 interacts with the R2R3 MYB transcription factors GLABARA1 (GL1) and MYB75 (Qi et al, 2011). These MYB transcription factors are components of the WD-repeat/bHLH/MYB complex which is an important regulatory machinery for anthocyanin accumulation and trichome development (Pesch & Hulskamp, 2009; Ramsay & Glover, 2005). Since JA is known to regulate these processes in a COI-dependant manner, these MYB TFs might be direct targets of JAZs and function

downstream of COI1 in the JA signaling. This is nicely verified by the result that overexpression of *GL1* in the *coi1-2* mutant plants can largely rescue trichome development and weakly rescue anthocyanin accumulation (Qi et al, 2011).

MYB21 and MYB24 are two additional R2R3 MYB transcription factors that interact with JAZ1, JAZ8 and JAZ11, and weakly interact with JAZ10. The *myb21 myb24* double mutant shows defects in pollen maturation, anther dehiscence, and filament elongation, which contributes to male sterility (Song et al, 2011). Furthermore, over-expression of *MYB21* in *coi1-1* mutant plants partially rescues the male sterile phenotype but not JA-regulated root growth, defense responses or anthocyanin accumulation (Song et al, 2011). These results demonstrate that different R2R3 MYB transcription factors mediate different downstream responses in JA signaling pathway.

JAZ interact with transcription factors involved in other hormone signaling pathways

Ethylene (ET) is an important plant hormone that works synergistically with JA to modulate plant defense against necrotrophic pathogens and regulate plant development processes (Wang et al, 2002). Recently, a crosstalk between the JA and ET hormone pathways that is mediated by JAZ was characterized. ETHYLENE INSENSITIVE 3 (EIN3), and its nearest homolog, EIN3-LIKE 1 (EIL1) are two important transcription factors that regulate ET-responsive gene expression (Guo & Ecker, 2004). EIN3 and EIL1 were also reported as positive regulators of a subset of

JA responses such as root development and plant defense (Zhu et al, 2011). JAZ1, JAZ3 and JAZ9 interact with EIN3/EIL1 and their interactions repress the functions of EIN3/EIL1. For example, ChIP-PCR assay showed that the in vivo association of EIN3 to the *ERF1* promoter was enhanced by JA or ET, which triggers the degradation of JAZs (Zhu et al, 2011). As such, JAZ degradation activates transcription factors in both JA and ET pathways, thus providing synergy between JA and ET in regulating defense responses

Besides ET signaling, JA pathway is integrated in a complex regulatory network which consists of a crosstalk with Gibberellin (GA) signaling (Cheng et al, 2009). Gibberellins are a group of plant hormones that regulate plant growth and multiple developmental processes, including germination, stem elongation, dormancy, flowering and fruit senescence (Yamaguchi, 2008). The *Arabidopsis* DELLA proteins including GAI, RGA, RGL1, RGL2 and RGL3 (Jiang & Fu, 2007), are repressors of GA signaling (Dill et al, 2001; Fu & Harberd, 2003; Silverstone et al, 2001). They repress the expressions of GA-induced genes in the absence of the hormone. GA relieves this repression via the receptor protein, GA-INSENSITIVE DWARF1 (GID1), which targets DELLA proteins for ubiquitination by the SCF^{SLY1} complex and the subsequent degradation via the 26S proteasome pathway (Gao et al, 2011). In addition to their roles in regulating GA signaling, DELLA proteins also impact the expression of JA-responsive genes (Peng, 2009). However, the molecular mechanisms underlying this phytohormone interaction remained elusive until recently, when a

yeast-two-hybrid screening identified interactions between the DELLA protein RGA with JAZ1, JAZ3 and JAZ9, via the ZIM domain of JAZs (Hou et al, 2010). Pull-down assays indicate that RGA and MYC2 compete for binding to JAZ1; as a result, RGA inhibits the interaction between JAZ1 and MYC2, thus enhances the activity of MYC2 to activate JA responsive genes (Hou et al, 2010). In the presence of GA, DELLA proteins are degraded, thus enabling the interaction between JAZ1 with MYC2, which blocks JA signaling (Hou et al, 2010).

Homo- or heteromeric interactions between JAZ proteins

Some JAZs, such as JAZ1, JAZ3, JAZ4 and JAZ9, form homodimers and heterodimers (Chini et al, 2009), although homomerization of full-length proteins was not observed for every JAZ. Detailed analyses using truncated JAZs indicated an important role of the ZIM domain in this homo- or heteromeric interactions between JAZ proteins (Chini et al, 2009). One possibility is that hetero-dimerization may stabilize JAZs. For example, the interaction between full length JAZs and JAZ3ΔJas inhibits the turnover of JAZs by COI (Chini et al, 2009; Chini et al, 2007). This would explain why over-expressing JAZ3ΔJas causes JA insensitivity in *Arabidopsis* (Chini et al, 2007).

JAZs interact with transcription co-repressors

Tandem affinity purification (TAP) is a technique that enables efficient isolation and identification of protein components in plant cells with relative low false positive rates. Using this technique, it was discovered that the NOVEL INTERACTOR OF JAZ (NINJA) protein forms complexes with JAZs (Pauwels et al, 2010). Yeast two-hybrid assay confirmed that NINJA interacts with most JAZs except for AtJAZ7 and AtJAZ8, which contain an asparagine residue in the fifth position of the TIFYXG motif. This residue is absent from the TIFY motif of all other JAZs. Further analysis showed that a region within the ZIM domain is sufficient for AtJAZ1-NINJA interaction.

NINJA over-expression in *Arabidopsis* leads to significant decrease in JA sensitivity (Pauwels et al, 2010). A subsequent TAP assay demonstrated that NINJA was also co-purified with the Groucho/Tup1 co-repressor TOPLESS (TPL) as well as its homologues TPR2 and TPR3, in the absent of JA (Pauwels et al, 2010). TPL and TPR proteins were reported to be involved in the regulation of auxin signaling. TPL physically interacts with IAA12/BODENLOS (IAA12/BDL) through an ETHYLENE RESPONSE FACTOR (ERF)–associated amphiphilic repression (EAR) motif and is required for the transcription repression activity of IAA12/BDL (Szemenyei et al, 2008). The interaction between NINJA with TPL suggests a transcription repressor activity of NINJA in JA signaling (Pauwels et al, 2010). This work provides mechanistic explanation of how JAZs function as negative regulators of JA signaling:

JAZ proteins recruit co-repressor NINJA and TPL/TPR to specific promoters via the interaction with MYC transcription factors, and inhibit the expression of JA-responsive genes (Pauwels et al, 2010; Wager & Browse, 2012).

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Chapter 1

HopZ1 Activates Jasmonate Signaling by Directly Targeting JAZ Transcriptional Repressors

(Shushu Jiang, Jian Yao, Ka-Wai Ma, Huanbin Zhou, Jikui Song, Sheng Yang He, and Wenbo Ma. 2013. *PLoS Pathogens 9(10), e1003715*)

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. My research show that the *Pseudomonas syringae* T3SE HopZ1, 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 effectors suggests that the JA receptor complex is potentially a major hub of host targets for bacterial pathogens.

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) (Zipfel, 2009). 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 (Boller & He, 2009; Jones & Dangl, 2006). However, successful pathogens produce virulence factors to effectively suppress PTI. For example, Gram-negative bacterial pathogens, such as *Pseudomonas syringae* (*P. syringae*), inject type III-secreted effectors (T3SEs) into the host cell to actively inhibit PTI (Block et al, 2008; Galan, 2009). 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 *effector-triggered immunity* (ETI), which is often associated with localized programmed cell death at the infection sites (Boller & He, 2009; Chisholm et al, 2006; Jones & Dangl, 2006).

Recent studies suggest that many *P. syringae* T3SEs suppress PTI and/or ETI by targeting important components of plant immunity (Galan, 2009; Grant et al, 2006;

Xin & He, 2013). 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/acetyltransferases produced by both plant and animal bacterial pathogens (Ma et al, 2006). 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 (Barrett & Rawlings, 2001). 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 Ikkα/β) in animal hosts and the acetylation of these host targets blocks their phosphorylation and the subsequent defense signal transduction (Mukherjee et al, 2006; Orth, 2002). PopP2 produced by the plant pathogen *Ralstonia solanacerum* 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 (Tasset et al, 2010).

Two functional HopZ1 alleles, HopZ1a and HopZ1b, have been identified in *P. syringae* (Ma et al, 2006). HopZ1b is produced by *P. syringae* pv. *glycinea* (*Pgy*) strains, which are the causal agents of bacterial blight disease on soybean (*Glycine max*) (Ma et al, 2006). HopZ1b$_{Pgy\text{BR1}}$ (HopZ1b in *Pgy* strain BR1; hereafter HopZ1b) promotes *P. syringae* multiplication in soybean; whereas the closely-related HopZ1a$_{Psy\text{A2}}$ (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) (Zhou et al, 2009). HopZ1 mutants with the catalytic

cysteine residues (Cys^{216} in HopZ1a or Cys^{212} 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 (Ma et al, 2006; Zhou et al, 2009). In addition, HopZ1 has a potential N-terminal myristoylation site (Gly^2) which directs the proteins to the plasma membrane (Lewis et al, 2008; Zhou et al, 2009). This myristoylation site of HopZ1a contributes to its avirulence function in both soybean and *Arabidopsis* (Lewis et al, 2008; Zhou et al, 2009). However, it is not clear whether this myristoylation site is important for the virulence function of HopZ1a. HopZ1 exhibited weak cysteine protease activities in vitro (Ma et al, 2006). 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 (Lee et al, 2012).

To identify potential host targets of HopZ1, my colleague Huanbin Zhou conducted yeast two-hybrid screening using a cDNA library of the natural host soybean and identified several HopZ1-interacting proteins (ZINPs). ZINP1 (2-hydroxyisoflavanone dehydratase, 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 (Zhou et al, 2011). 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 HopZ1 in *Arabidopsis*, I 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 (Chini et al, 2007; Sheard et al, 2010; Thines et al, 2007). JA plays an important role in regulating plant responses to biotic and abiotic stresses. Some *P. syringae* strains produce the JA-mimicking phytotoxin coronatine, which efficiently activates JA signaling to facilitate bacterial entry into plant apoplastic space and suppress defense (Brooks et al, 2005; Katsir et al, 2008; Zeng et al, 2011). 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 *AtPDF1.2* in *Arabidopsis*, indicating that it could activate JA/ethylene signaling (Macho et al, 2010).

Here, I report that HopZ1 directly interacts with JAZ proteins of soybean and *Arabidopsis*. My results 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, my research suggests that HopZ1 facilitates bacterial infection by manipulating the JA signaling pathway in soybean and *Arabidopsis*.

MATERIALS AND METHODS

Bacterial strains and plasmids

Bacteria strains and plasmids used in this study are summarized in Table 1.1. *Agrobacterium tumefaciens* (*A. tumefaciens*) and *Escherichia coli* (*E. coli*) were grown in Luria–Bertani (LB) medium (BD Co., Franklin Lakes, NJ, USA) at 30°C

and 37°C respectively. Plasmids were transformed into *A. tumefaciens* and *E. coli* chemical competent cells as previously described (Morgan et al, 2010). Appropriate antibiotics were used in bacterial growth media when necessary. *Pseudomonas syringae* strains were grown at 30°C in King's B medium (King et al, 1954) or M63 minimal medium containing 1% fructose for the induction of T3SS as reported (Bretz & Hutcheson, 2004).

P. syringae transformation was conducted using an Eppendorf electroporator 2510 (Eppendorf North America, Westbury, NY, USA) according to the manufacturer's instructions. Briefly, 1 mL of the overnight *P. syringae* culture was used to inoculate 4 mL of King's B medium with appropriate antibiotics. 1 mL of cell culture was then collected for each transformation before the bacterial density reach OD_{600}=1. Cell pellets were washed with 0.5 M sucrose for three times (1mL, 0.8 mL and 0.8 mL of sucrose for each time) and then resuspended in 100 μL 0.5 M sucrose with 1 μL plasmid of interest. The cell mixtures were then transferred to the pre-cold cuvette (Eppendorf) with its outside walls completely dried. The transformation was performed by double clicking on the start button using 2,500 volts, and the whole process usually takes more than five seconds. 1 mL of antibiotics-free King's B medium was immediately added into the cuvette for bacterial growing. After growing in the eppendorf tube at 30°C for more than 1 hour, the cell culture was spread on the plate and grown for 2 days before PCR detection.

Table 1.1. Bacterial strains and plasmids used in the study in Chapter 1.

Strains or Plasmids	Description	Source/ reference
Escherichia coli DH5a	F- Φ80d*lacZ*ΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1, *hsd*R17(rk-, mk+) *pho*A *sup*E44 λ- *thi*-1 *gyr*A96 *rel*A1	Invitrogen
Escherichia coli BL21(DE3)	F^- *ompT gal dcm lon hsd*S_B(r_B^- m_B^-) λ(DE3 [*lacI lacUV5-T7 gene 1 ind1 sam7 nin5*]	Invitrogen
Pseudomonas syringae pv. *glycinea* strain BR1Rif-O1	Endogenous plasmid (harboring *hopZ1b*)-cured line of *Pseudomonas syringae* pv. glycinea strain BR1Rif, Rif^R	Zhou et al., 2009
Pseudomonas syringae pv. *tomato* strain DC3000	Isolated from tomato plants, also infects *Arabidopsis*, Rif^R	Cuppels, 1986
Pseudomonas syringae pv. *tomato* strain DC3118	A mutant of *Pto*DC3000 that does not produce coronatine, Rif^R, Kan^R	Melloto et al., 2006; Moore et al, 1989
Agrobacterium tumefaciens GV3101(pMP90)	Rif^R, $Gent^R$	Holsters, 1980
Agrobacterium tumefaciens C58C1 (pCH32)	Rif^R, Tet^R	Mudgett et al., 2000
pUCP18	Plasmid vector multiplies in *P. syringae*, Amp^R	Schweizer, 1991
pUCP18::*HopZ1a-HA*	pUCP18 carrying the gene encoding HopZ1a tagged with HA and under the control of the native promoter, Amp^R	This study
pUCP18::*HopZ1a(C216A)-HA*	pUCP18 carrying the gene encoding the HopZ1a catalytic mutant with Cys216 replaced with an alanine, Amp^R	This study
pUCP18::*HopZ1a(G2A)-HA*	pUCP18 carrying the gene encoding the HopZ1a mutant with Gly2 replaced with an alanine, Amp^R	This study
pDSK600::*avrRpt2*	pDSK600 carrying *avrRpt2* gene under the control of its own promoter, Rif^R, Kan^R	Mudgett and Staskawicz, 1999
pUCP20tk	Plasmid vector multiplies in *P. syringae*, Kan^R	Zhou et al., 2009
pUCP20tk::*hopZ1a-HA*	pUCP20tk carrying the gene encoding HopZ1a tagged with HA and under the control of the native promoter, Kan^R	Ma et al., 2006
pUCP20tk::*hopZ1a(C216A)-HA*	pUCP20tk carrying the gene encoding the HopZ1a catalytic mutant with Cys216 replaced with an alanine, Kan^R	Zhou et al., 2009
pUCP20tk::*hopZ1b-HA*	pUCP20tk carrying the gene encoding HopZ1b tagged with HA and under the control of its native promoter, Kan^R	Zhou et al., 2009
pUCP20tk::*hopZ1b(C212A)-HA*	pUCP20tk carrying the gene encoding the HopZ1b catalytic mutant with Cys212 replaced with an alanine, Kan^R	Zhou et al., 2009
pMDD1	A binary vector with cauliflower mosaic virus *35S* promoter, Kan^R	Mudgett et al., 2000
pMDD1::*hopZ1a-HA*	pMDD1 carrying *hopZ1a* tagged with HA, Kan^R	Zhou et al., 2009
pMDD1::*hopZ1b-HA*	pMDD1 carrying *hopZ1b* tagged with HA, Kan^R	Zhou et al., 2009
pMDD1::*hopZ1a(C216A)-HA*	pMDD1 carrying *hopZ1a(C216A)* tagged with HA, Kan^R	Zhou et al., 2009
pMDD1::*hopZ1b(C212A)-HA*	pMDD1 carrying *hopZ1b(C212A)* tagged with HA, Kan^R	Zhou et al., 2009
pMD1::*avrRpt2-HA*	pMD1 carrying avrRpt2tagged with HA, KanR	Gitta Coaker
pEG100	pEarleyGate100, a Gateway binary vector with cauliflower mosaic virus *35S* promoter, Kan^R	Earley et al., 2006
pEG100::*GmJAZ1-FLAG*	pEG100 carrying *GmJAZ1* tagged with FLAG at the C-terminus, Kan^R	This study
pEG100::*3×FLAG-HopZ1a*	pEG100 carrying *hopZ1a* tagged with 3×FLAG at N-terminus, Kan^R	This study

pEG100::3×*FLAG-HopZ1a(C216A)*	pEG100 carrying *hopZ1a(C216A)*tagged with 3×FLAG at N-terminus, Kan^R	This study
pEG101	pEarleyGate101, a Gateway binary vector for YFP fusion protein expression with cauliflower mosaic virus *35S* promoter, Kan^R	Earley et al., 2006
pEG101::*GmJAZ1*	*GmJAZ1* is in-frame fused to YFP and HA, Kan^R	This study
pEG101::*AtJAZ6*	*AtJAZ6* is in-frame fused to YFP and HA, Kan^R	This study
pEG101::*AtJAZ6ΔJas*	*AtJAZ6ΔJas* (with 10 aa deletion in the Jas domain) is in-frame fused to YFP and HA, Kan^R	This study
pSPYNE	A binary vector with cauliflower mosaic virus *35S* promoter and the N-terminal (1-155 aa) domain of YFP (nYFP), Kan^R	Walter et al., 2004
pSPYCE	A binary vector with cauliflower mosaic virus 35s promoter and the C-terminal (156-239 aa) domain of YFP (cYFP), Kan^R	Walter et al., 2004
pSPYNE::*hopZ1a(C216A)*	pSPYNE carrying *hopZ1a(C216A)* in-frame fused with nYFP, Kan^R	Zhou et al., 2011
pSPYNE::*hopZ1b(C212A)*	pSPYNE carrying *hopZ1b(C212A)* in-frame fused with nYFP, Kan^R	Zhou et al., 2011
pSPYCE::*GmJAZ1*	pSPYCE carrying *GmJAZ1* in-frame fused with cYFP, Kan^R	This study
pSPYCE::*AtJAZ6*	pSPYCE carrying *AtJAZ6* in-frame fused with cYFP, Kan^R	This study
pGEX4T-2	*E. coli* expression vector with an N-terminal GST tag, Amp^R	Amersham
pGEX4T-2::*hopZ1a*	pGEX4T-2 carrying *hopZ1a*, Amp^R	Zhou et al., 2011
pGEX4T-2::*hopZ1b*	pGEX4T-2 carrying *hopZ1b*, Amp^R	Zhou et al., 2011
pGEX4T-2::*hopZ1a(C216A)*	pGEX4T-2 carrying *hopZ1a(C216A)*, Amp^R	This study
pGEX4T-2::*hopZ1a T1*	pGEX4T-2 carrying a truncated *hopZ1a* which encodes amino acids 1-62, Amp^R	This study
pGEX4T-2::*hopZ1a T2*	pGEX4T-2 carrying a truncated *hopZ1a* which encodes amino acids 63-244, Amp^R	This study
pGEX4T-2::*hopZ1a T3*	pGEX4T-2 carrying a truncated *hopZ1a* which encodes amino acids 245-370, Amp^R	This study
pGEX4T-2::*hopZ1a T4*	pGEX4T-2 carrying a truncated *hopZ1a* which encodes amino acids 1-244, Amp^R	This study
pGEX4T-2::*hopZ1a T5*	pGEX4T-2 carrying a truncated *hopZ1a* which encodes amino acids 63-370, Amp^R	This study
pET14b	*E. coli* expression vector with an N-terminal 6×His tag, Amp^R	Novagen
pET14b::*GmJAZ1*	pET14b carrying *GmJAZ1* with an N-terminal 6×His tag, Amp^R	This study
pET14b::*GmJAZ1 T1*	pET14b carrying a truncated *GmJAZ1* which encodes amino acids 45-168, Amp^R	This study
pET14b::*GmJAZ1 T2*	pET14b carrying a truncated *GmJAZ1* which encodes amino acids 1-168, Amp^R	This study
pET14b::*GmJAZ1 T3*	pET14b carrying a truncated *GmJAZ1* which encodes amino acids 45-233, Amp^R	This study
pET14b::*GmJAZ1ΔZIM*	pET14b carrying a truncated *GmJAZ1* which encodes a GmJAZ1 mutant with the amino acids 78-152 deleted, Amp^R	This study
pET-mal	*malE* gene from pMAL-c2 is cloned into *Nde*I-*Xho*I site of pET28a, Kan^R	Sweeney et al., 2005
pET-mal::*AtJAZ*	pET-mal carrying *AtJAZ* genes, Kan^R	Sheng Yang He
pET-mal::*AtJAZ6ΔJas*	pET-mal carrying *AtJAZ6ΔJas* (with 10 aa deletion in the Jas domain), Kan^R	This study
pENTR/D-TOPO	An entry vector for the Gateway system, Kan^R	Invitrogen
pENTR/D-TOPO::3×*FLAG-hopZ1a*	pENTR/D-TOPO carrying the *hopZ1a* gene with a 3×FLAG tag at the N-terminus, Kan^R	This study
pENTR/D-TOPO::3×*FLAG-hopZ1a(C216A)*	pENTR/D-TOPO carrying the *hopZ1a(C216A)* gene with a 3×FLAG tag at the N-terminus, Kan^R	This study
pSITE2CA	binary vector, carrying the *GFP* gene, Kan^R	Chakrabarty et al., 2007
pSITE2CA::*AtJAZ6*	pSITE2CA carrying AtJAZ6 to make a N-terminal fusion of AtJAZ6 with GFP, Kan^R	Sheng Yang He

pPH4A-GW-Venus ::*HopZ1a(C216A)*	T-DNA binary vector pPH4A-GW-Venus carrying *HopZ1a(C216A)*	Sheng Yang He
pJYP003::*3×HA-AtJAZ6*	P_{35S} carrying *AtJAZ6* with a 3×HA tag at the N-terminus	Yang et al., 2012

Fluorescence microscopy

To construct plasmids for bimolecular fluorescence complementation (BiFC) assay, full-length cDNAs of *GmJAZ1* or *AtJAZ6* were cloned into the vector pSPYCE, and *hopZ1a(C216A)* or *hopZ1b(C212A)* were cloned into the vector pSPYNE (Walter et al, 2004). pSPYCE and pSPYNE carry the C-terminal half (156-239aa) or the N-terminal half (1-155aa) of yellow fluorescence protein (YFP), respectively. The recombinant plasmids were introduced into *A. tumefaciens* strain C58C1(pCH32) (Mudgett et al, 2000). The *Agrobacterium* strain expressing P19, a viral suppressor of RNA silencing from tomato bushy stunt tombusvirus (Chu et al, 2000), was used to infiltrate *Nicotiana benthamiana* together with *Agrobcterium* strains carrying HopZ1 or JAZs.

Basically, single *Agrobacterium* colony on LB plates was inoculated into 5 mL liquid LB medium supplemented with the appropriate antibiotics and grown at 30°C for 24-48 hours. 100 μL of the cell culture was transferred into 5 mL LB medium with 100 μL 0.5 M MES and 2 μL 100 mM acetosyringone, and then induced at 30°C for 16 hours. On the next day, the bacteria pellets were collected and resuspended in an infiltration buffer (10 mM $MgCl_2$, 10 mM MES, 0.15 mM acetosyringone). The densities of bacterial suspensions expression HopZ1 or JAZs were adjusted to an OD_{600}=1.5, and cells expressing P19 were resuspended in the infiltration buffer at an OD_{600}=1. Equal volume of *Agrobacterium* suspensions expressing HopZ1, JAZ, or P19 were mixed together and used to infiltrates leaves of 3-4 week old *N. benthamiana* plants using a needle-less syringe.

To examine the subcellular localization of GmJAZ1 and AtJAZ6, full-length cDNA of *GmJAZ1* was cloned into the vector pEG101 (Earley et al, 2006).

pSITE2CA::*AtJAZ6* (Chakrabarty et al, 2007) was obtained from Dr. Sheng Yang He at Michigan State University. The recombinant plasmids were introduced into *A. tumefaciens* strain C58C1(pCH32), which were then used to infiltrate 3-4 week old *N. benthamiana* plants at a final OD_{600}=0.5.

The inoculated plants were kept at room temperature under continuous low light. Functional fluorophore were visualized in the infiltrated leaves using a Leica SP2 Laser Scanning Confocal Microscope (Leica Microsystems) at 48 hours post inoculation (hpi) for subcellular localization and BiFC.

To visualize the nuclei in plant cells, leaf tissues were stained with 4', 6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI, Life technologies) (James & Jope, 1978; Kapuscinski & Skoczylas, 1977). The DAPI stock solution was prepared in a 1.5 mL eppendorf tube at the concentration of 5 mg/mL in ddH_2O. The tube was wrapped with aluminum foil and stored in -20°C. A 1:1000 dilution of the stock solution was made in 1×PBS solution and used to infiltrate *N. benthamiana* leaves about 30 minutes before confocal detection.

In vitro GST pull-down assays

To construct GST-fusion proteins, the full-length *hopZ1a* and *hopZ1b* genes were inserted into the vector pGEX4T-2 (GE Healthcare Life Science). *GmJAZ1-HA* gene was cloned into the vector pET14b (Navogen), which has 6×His in the N-terminus. The *AtJAZ* 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 (Sweeney et al, 2005) provided by Dr. Sheng Yang He at Michigan State University. The recombinant plasmids were then introduced into *E. coli* strain BL21(DE3).

Large-scale protein induction protocol was applied to the inductions of GST, HopZ1 or AtJAZs. Overnight bacterial cultures were used to inoculate 500 mL or 1 L LB medium at an inoculum of 1%. When the bacterial density reached OD_{600}=0.6, which usually took around 2-3 hours, IPTG (final concentration of 2 mM) was added to induce protein production. The induced cells were collected after 8-20 hours for protein purification.

GmJAZ1 was induced using a small-scale protocol because of the low induction efficiency of this protein using the above-described procedure. 100 µL of overnight bacterial culture was used to inoculate 5 mL LB medium in a test tube. IPTG was added into the tubes at a final concentration of 5 mM when the bacterial density reached OD_{600}=0.5-0.6. The induced cells were collected 4-5 hours after IPTG induction.

In vitro pull-down assays were carried out using ProFound™ pull-down GST protein:protein interaction kit (Pierce) according to the manufacturer's instruction. Briefly, induced *E. coli* cells expressing GST, GST-HopZ1 or JAZs were homogenized by sonication using Cell Disruptor 350 (Branson Sonic Power Co.) at power setting 5 and 50% duty cycle. All the cells were sonicated for twenty pulsed times in one cycle for 4-5 cycles with more than one minute gap between each cycle. And then cell debris was spun down at 12,000 rpm for 90 minutes. After that, soluble proteins were transferred and incubated with 50 µL glutathione agarose beads (Invitrogen) for one hour at 4°C. The beads were washed five times in a washing buffer (20 mM Tris-HCl (pH=7.5), 150 mM KCl, 0.1 mM EDTA and 0.05% Triton X-100) and then incubated with equal amount of bacterial lysates containing JAZ proteins or purified JAZs 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.).

HIS- tagged Protein purification

The IPTG-induced *E. coli* cell pellets were resuspended in a lysis buffer (50 mM Tris-HCl (pH 8.0), 1 M NaCl, 20 mM immidazole, 200 μM PMSF, 500 μM DTT) and subjected to sonication. The clear supernatants, containing soluble proteins, were transferred to a new tube and incubated with 500 μL nickel resins (Ni-NTA agarose, Qiagen) for at least one hour at 4°C before they were removed by gravity without centrifugation. After five times of washing with a washing buffer (50 mM Tris-HCl (pH=8.0), 1 M NaCl, 20 mM immidazole), the resins were incubated with the elution buffer (50 mM Tris-HCl (pH=8.0), 1 M NaCl, 200 mM immidazole) to elute the purified HIS-tagged proteins.

Type III effector induction in P. syringae

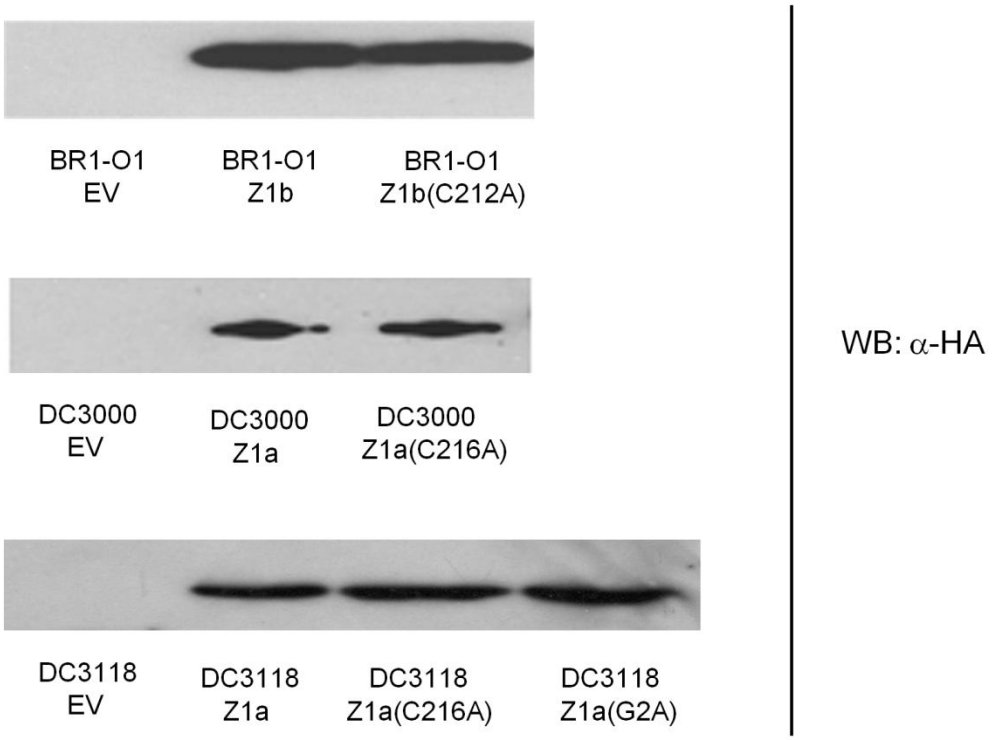
P. syringae carrying the *hopZ1-HA* genes were grown in King's B medium with appropriate antibiotics at 30°C overnight. The bacteria cells were collected by centrifugation at 4000 rpm for five minutes and washed once with M63 minimal medium (Morgan et al, 2010). The cells were then resuspended in M63 medium containing 1% fructose at the OD_{600} of 0.5 (approximately 5×10^8 cfu/mL), and induced with agitation overnight at room temperature. Cells from 1 mL of the induced culture were collected and expressions of HopZ1a and its mutants in *P. syringae* pv. *tomato* (*Pto*) strain DC3000 or *Pto*DC3118 as well as the expressions of HopZ1b and

its catalytic mutant in *P. syringae* pv. *glycinea* (*Pgy*) strain BR1-01 were detected by western blots using the anti-HA antibody (Figure 1.1).

Figure 1.1. HopZ1 expression in *Pgy*BR1-O1, *Pto*DC3000 or *Pto*DC3118 cells was demonstrated by western blots to confirm that differences in the cellular function of the wild type and the catalytic mutant of HopZ1 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 proteins were detected by western blots using anti-HA antibody.

These experiments were repeated twice with similar results.



JAZ degradation assay by HopZ1

The degradation of JAZ proteins by HopZ1 was examined using three systems: 1) co-transient expression of JAZ and HopZ1 in *N. benthamiana*; 2) semi in vitro degradation assay using proteins extracted from *N. benthamiana* tissues that transiently expressed JAZ or HopZ1, individually; 3) in vivo JAZ degradation assay of *Arabidopsis* inoculated with *P. syringae* expressing HopZ1a.

For JAZ degradation assay in *N. benthamiana*, all the plasmids were transformed into *A. tumefaciens* strain GV3101(pMP90) (Holsters et al, 1980). HopZ1a-HA or 3×FLAG-HopZ1a were co-expressed with GmJAZ1-FLAG or AtJAZ6-YFP-HA using *Agrobacterium*-mediated transient expression as previously described (Ma et al, 2006; Szurek et al, 2001). Leaf disks were collected at 20 hpi, and then grounded in 2×Laemmli buffer (0.125 M Tris-HCl (PH 6.8), 10% (v/v) β-mercaptoethanol, 4% (w/v) SDS, 20% (v/v) glycerol and 0.04% (w/v) bromophenol blue). 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 transiently expressed in *N. benthamiana* using *Agro*-inoculation, individually. Total proteins were extracted from the infected leaf tissues at 20 hpi. The ground leaf powders were homogenized using an equal volume of 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 then mixed in equal volume and incubated for six hours at 4°C with gentle agitation. The abundance of GmJAZ1 proteins was then examined by western blots. AvrRpt2 was used as a negative control. The pMD1::a*vrRpt2-HA* construct used in this study was provided by Dr. Gitta Coaker at UC Davis.

For the in vivo JAZ degradation assay, six-week-old *35S-HA-AtJAZ1* transgenic *Arabidopsis* plants (seeds obtained from Dr. Sheng Yang He at Michigan State University) were hand-infiltrated with *Pto*DC3000 (Cuppels, 1986) or its mutant strain *Pto*DC3118 (Moore et al, 1989) carrying the pUCP18 empty vector (EV) (Schweizer, 1991), pUCP18:: *HopZ1a-HA*, pUCP18 :: *HopZ1a(C216A)-HA* or pDSK600::*avrRpt2-HA* (Mudgett & Staskawicz, 1999) at OD_{600}=0.2 (approximately 2×10^8 cfu/mL) in 10 mM $MgSO_4$. Leaves infiltrated with water were used as a negative control. Six hours post inoculation, total proteins were extracted from four leaf discs (0.5 cm^2) in 100 µL of 2×Laemmli buffer. The lysates were incubated at 95°C for 10 minutes followed by centrifugation at 14,000 rpm for 5 minutes. The abundances of AtJAZs were then analyzed by western blots.

In vivo JAZ degradation assay was also performed in *Arabidopsis* plants expressing *35S-HA-AtJAZ1* in *the coi1-30* background (seeds obtained from Dr. Sheng Yang He at Michigan State University). Because the homozygous *coi1* mutants are male-sterile (Benedetti et al, 1995; Feys et al, 1994), 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

HopZ1a, JAZs and their mutants were cloned in plasmid vector pRSF, which introduces 8× HIS and SUMO tags at N-terminus of the proteins (provided by Dr. Jikui Song in the Department of Biochemistry at UC, Riverside) or pET-MAL

(Sweeney et al, 2005). HIS-GmJAZ1, HIS-SUMO-HopZ1a, HIS-SUMO-HopZ1a(C216A), HIS-SUMO-AtJAZ6, MBP-AtJAZ6-HIS, and MBP-AtJAZ6ΔJas-HIS were expressed in the *E. coli* strain BL21(DE3) and then purified using nickel resins. HIS-SUMO-HopZ1a, HIS-SUMO-HopZ1a(C216A), and HIS-SUMO-AtJAZ6 proteins were cleaved using ubiquitin like protease 1 (ULP1), producing protein mixtures with both the HIS-SUMO tag and tag-free HopZ1a or AtJAZ6. The protein mixtures were incubated with nickel resins again, which bind the HIS-SUMO peptides, and the tag-free HopZ1a or AtJAZ6 proteins were collected from the flow through. Purified proteins were dialysed into a low salt solution (20 mM Tris-HCl (pH=7.5), 200 mM NaCl, 5 mM DTT or β-mercaptethanol) overnight at 4°C and stored with glycerol at a final concentration of 10% at -80°C.

In a standard acetylation assay, 2 μg purified HopZ1a or HopZ1a(C216A) proteins were incubated with 10 μg MBP-AtJAZ6, 5 μg AtJAZ6 or 1 μg GmJAZ1 at 30°C for one hour in a 25 μL reaction system, which consists 50 mM HEPES (pH=8.0), 10% glycerol, 1 mM DTT, 1 mM PMSF, 10 mM sodium butyrate, 1 μL [14C]-acetyl-CoA (55 μci/μmol, Amersham) and 100 nM IP6 (Sigma). The reaction mixtures were then subjected to SDS-PAGE and the acetylated proteins were detected by autoradiography as previously described (Mittal et al, 2006; Mittal et al, 2010; Mukherjee et al, 2006) 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 *AtJAZ9*, *AtJAZ10* and *AtICS1* in *Arabidopsis* adult leaves as well as *GmPR3* and *GmVSPA* in soybean unifoliates were analyzed by real-

time RT-PCR using SYBR Green Supermix and a CFX96 Real-Time PCR Detection System (BioRad Laboratories). Five-week old *zar1-1* or *coi1-1, zar1-1* (generated by Mr. Ka-Wai Ma) *Arabidopsis* plants were hand-inoculated with *Pto*DC3118 expressing HopZ1a or HopZ1a(C216A) at OD_{600} = 0.2.

Soybean seeds were surface sterilized using 10% bleach for 10 min and then pre-germinated between two wet filter papers in a petri dish at room temperature in the dark for 4 – 5 days. After that, the seedlings were transplanted into soil and grown at 25 - 30°C for around nine days until unifoliates are fully expanded. The cell suspensions of *Pgy* BR1-O1 carrying the empty vector, HopZ1b or HopZ1b(C212A) were used to spray the unifolicates at an OD_{600}=0.2 using a commercial air brush and an air compressor (approximately 40 psi).

Total RNA was isolated from four *Arabidopsis* leaves at 6 hpi or 100 mg of soybean unifoliates at 9 hpi using 1 mL Trizol. Genomic DNA was removed from the RNA prep using DNase I following product instructions (Fermentas). Reverse transcription was performed using M-MLV Reverse Transcriptase (Promega) with 1 μg of total RNA in a 25 μL reaction system. The synthesized cDNA then served as templates for real-time PCR using gene-specific primers, which are listed below. *AtActin* and *AtUBQ5* were internal controls used for the normalization of gene expression levels in *Arabidopsis*. *GmUBI* was used as the internal standard for soybean gene expression analysis.

AtActin: 5’-GGTGTCATGGTTGGTATGGGTC-3’ and

5’-CCTCTGTGAGTAGAACTGGGTGC-3’

AtJAZ9: 5’-ATGAGGTTAACGATGATGCTG-3’ and

5’-CTTAGCCTCCTGGAAATCTG-3’

AtJAZ10: 5’-GTAGTTTCCGAGATATTCAAGGTG-3’ and

5’-GAACCGAACGAGATTTAGCC-3’

AtUBQ5: 5’-GACGCTTCATCTCGTCC-3’ and

5’-GTAAACGTAGGTGAGTCC-3’

AtICS1: 5’-GGCAGGGAGACTTACG-3’ and

5’-AGGTCCCGCATACATT-3’

GmUBI: 5’-TGTGTAATGTTGGATGTGTTCCC-3’and

5’-ACACAATTGAGTTCAACACAAACC-3’

GmPR3: 5’-GTTCGGTGATGCTATGCTTA-3’ and

5’-TGTTGGAGTTGGTGTTGAG-3’

GmVSPA: 5’-CTCCGAATGAACACTGGCTATG-3’ and

5’-CAACGCACTCTTCAGGAATGG-3’

In planta bacterial multiplication assays

Arabidopsis mutants (*zar1-1* or *coi1-1*, *zar1-1*) were grown in a controlled growth chamber at 22°C with a 12 hour photoperiod under light intensity of 100 mE/m^2/s as previous described (Lewis et al, 2010; Melotto et al, 2006). The leaves of five-week old plants were dipped into the *P. syringae* bacterial suspensions at an OD_{600} = 0.2 for 15 seconds. The inoculated plants were then transferred to a growth chamber (22°C and 16/8 light/dark regime, 90% humidity) for three days. To determine the *in planta* bacteria populations, four leaf discs (0.5 cm^2) were taken from each inoculated plant at 0 and 3 days post inoculation (dpi). The leaf disks were briefly surface sterilized in 70% ethanol and then homogenized in sterile 10 mM $MgSO_4$. Serial dilutions of the tissue suspension were plated onto King’s B agar with appropriate antibiotics and

incubated at 30°C for 2 days to determine the colony forming units (cfu) (Morgan et al, 2010).

Statistical analysis

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

Transgenic plants generation

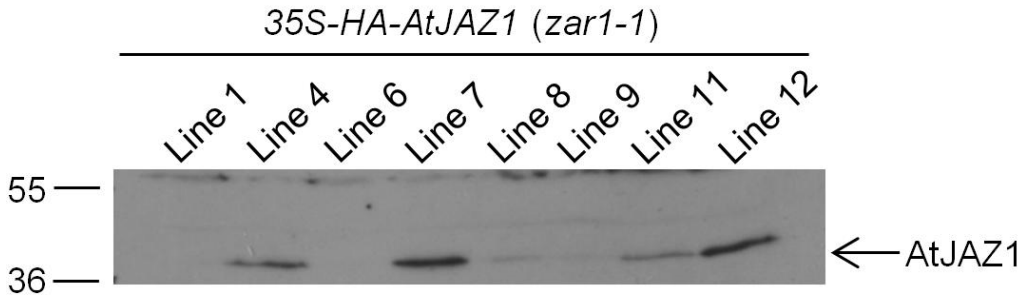
Single *Agrobacterium* colony was selected to grow in 5 mL of liquid LB medium with appropriate antibiotics for 24 - 48 hours. One day before infiltration, 500 μL bacterial suspensions from this culture were inoculated into 500 mL LB medium. After 12 hours of growth, the cell densities usually reached OD_{600}=1. Then the cells were harvested by centrifugation and resuspended in the infiltration buffer (1/2 MS salt with 5% sucrose and 0.03% Silwet-77, pH=5.7) at a final OD_{600}=0.8. The *Arabidopsis* flowers were dipped into the bacterial suspension for 10 min. After dipping inoculation, the plant pots were laid on one side of a flat and covered with plastic wrap that was sprayed with water in the inner side to keep high humidity. The plants were left in the dark for one day. On the next day, the plants were sprayed with water to remove the remaining silwet-77 on the flowers and then were placed upright for growing. After the seeds were collected from the inoculated flowers and grown in the soil, basta herbicide was applied to select for seedlings carrying the transgene. Total DNAs and proteins were extracted from the leaves of the 3-4 week potential transgenic plants for PCR and western blots to confirm the gene insertion and protein expression.

The homozygous transgenic *35S-HA-AtJAZ1 Arabidopsis* plants in the wild type Col-0 background were obtained from Dr. Sheng Yang He at Michigan State University. Heterozygous *35S-HA-AtJAZ1* transgenic plants in the *coi1-30* background were generated by Dr. Jian Yao at Michigan State University. Homozygous *35S-HA-AtJAZ1* transgenic plants were also constructed in *zar1-1* mutant background using the recombinant plasmid pJYP003::*3×HA-AtJAZ1* provided by Dr. Jian Yao. Western blotting was used to confirm the *AtJAZ1* expression from independent transgenic lines (Figure 1.2).

Figure 1.2. Four independant lines of *35S-HA-AtJAZ1* transgenic *Arabidopsis* plants can strongly express *AtJAZ1*.

Eight lines of *35S-AtJAZ1* homozygous transgenic plants in *zar1-1* mutant background were selected for western blots detection of *AtJAZ1* expression using anti-HA antibody.

This experiment was repeated twice times with similar results.



Gene cloning and plasmid construction

For the in vitro pull-down assay, *GmJAZ1*, *GmJAZ1-T1*, *GmJAZ1-T2*, *GmJAZ1-T3* were cloned into pET-14b vector using *Nde*I and *Bam*HI digestion sites. pET-14b::*GmJAZ1*Δ*Jas* was constructed by ligating a linear PCR product using pET-14b::*GmJAZ1* as the template and a pair of primers designed from the two ends of the deleted region. *HopZ1a* and *HopZ1a(C216A)* were fused with DNA sequence that encodes the GST tag in the pGEX-4T2 vector into the *Bam*HI digestion site. All the genes that encode truncated HopZ1a were cloned in the same vector using the *Bam*HI and *Xho*I digestion sites.

pET-MAL::*AtJAZ* constructs were provided by Dr. Sheng Yang He at Michigan State University. pET-MAL::*AtJAZ6*Δ*Jas* was constructed by ligating a linear PCR product using pET-MAL::*AtJAZ6* as the template and a pair of primers designed from the two ends of the deleted region. For the BiFC assay, pSPYCE::*GmJAZ1* was constructed by my colleague Dr. Huanbin Zhou.

pSPYCE::*AtJAZ6* was obtained by inserting *AtJAZ6* into the pSPYCE vector at the *Bam*HI and *Xho*I digestion sites.

For protein purifications and acetylation assays, *Bam*HI and *Xho*I digestion sites in pRSF vector were used to make all the constructs. Genes encode HopZ1a truncate (31-369aa) or HopZ1b truncate (31-368aa) were cloned and fused with 6x HIS and SUMO in the pRSF vector. These truncates were used for acetylation assays because they have better yields and the N-terminal regions of HopZ1 do not contain secondary structures according to protein secondary structure analysis (data not shown, conducted with Dr. Jikui Song from the Department of Biochemistry at UCR). Besides, these truncates showed exactly the same enzymatic activity as the full length

HopZ1 according to my experiment (data not shown). HopZ1 catalytic mutants or chimeric proteins used in the acetylation assays were all truncated forms starting from the 31st amino acid. Except for HopZ1, all the other purified proteins used in acetylation assays are full-length proteins.

To construct pUCP18::*HopZ1a-HA*, pUCP18::*HopZ1a(C216A)-HA* and pUCP18::*HopZ1a(G2A)-HA* for the bacterial growth assays, *HopZ1a* wild type and mutant genes with the native promoter were cut out from the corresponding pUCP20tk::*HopZ1a* constructs using *Bam*HI and *Kpn*I and inserted into pUCP18.

For the *Agrobacterium*-mediated transient expression assays in *N. benthamiana*, *GmJAZ1-6×HIS-FLAG* was inserted into the vector pENTR1a at the *Bam*HI and *Xho*I digestion sites. The Gateway recombinational cloning reactions were performed as described by the manufacturer (Invitrogen) to obtain the construct pEG100::*GmJAZ1-HIS-FLAG*. In addition, via *Bam*HI and *Xho*I sites, both *GmJAZ1* and *AtJAZ6* genes with two nucleotides added in the C-termini were inserted into pENTR1a to make sure that these genes would be in-frame fused with DNA sequences encoding the YFP and HA epitopes in the pEG101 destination vector after Gateway reaction. *3×FLAG-hopZ1a* and *3×FLAG-hopZ1a(C216A)* were cloned into a modified pENTR/D-TOPO vector (obtained from Dr. Shengben Li at UC, Riveside) at the *Bam*HI and *Xba*I sites and finally exchanged into pEG100.

RESULTS

HopZ1 physically interacts with GmJAZ1

Using yeast two-hybrid screens, HopZ1a-interacting proteins (ZINPs) were identified from a soybean cDNA library (Zhou et al, 2011). Some of these targets have been characterized and reported earlier (Zhou et al, 2011). Among them, ZINP3 (Gm7g04630) was interesting to me 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* as well as SlJAZ1 in tomato (Figure 1.3). In this thesis, I pursued GmJAZ1 and AtJAZ proteins as potential direct targets of HopZ1 in plants.

I first confirmed the physical interaction between HopZ1 and GmJAZ1 by in vitro pull-down using recombinant GST-HopZ1 and GmJAZ1-HA proteins over-expressed in *E. coli*. GST-HopZ1a, GST-HopZ1b or GST (empty vector) were purified from whole cell lysate using glutathione resins and then incubated with an equal amount of whole cell lysate expressing GmJAZ1-HA. Both GST-HopZ1a- and GST-HopZ1b-bound resins, but not GST-bound resins, provided enrichment of GmJAZ1-HA (Figure 1.4), suggesting that HopZ1a and HopZ1b interacted with GmJAZ1 in vitro. The catalytic mutant HopZ1a(C216A) also interacted with GmJAZ1, similar to wild-type HopZ1a (Figure 1.4). This experiment demonstrated that GmJAZ1 interacts with both HopZ1a and HopZ1b, and this interaction does not require the enzymatic activity of HopZ1.

Figure 1.3. Sequence alignment of three JAZ1 homologs from *Arabidopsis* (At), soybean (Gm) and tomato (Sl).

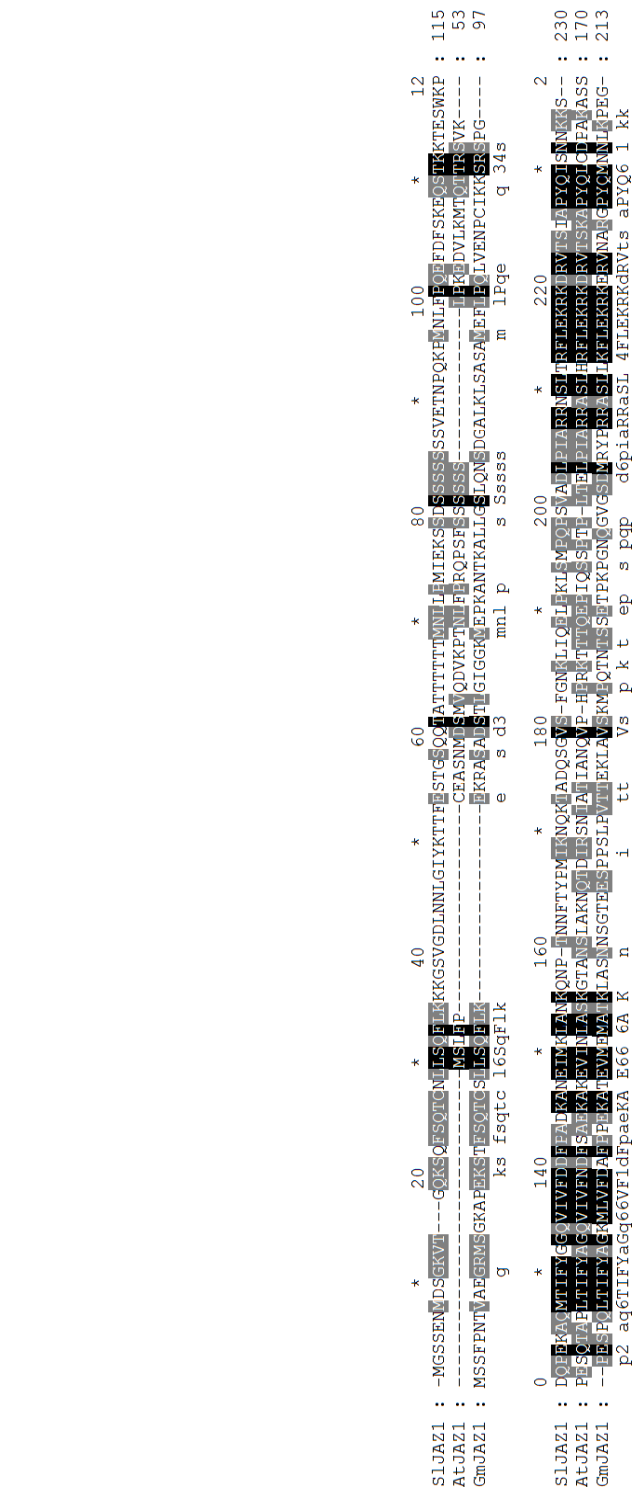
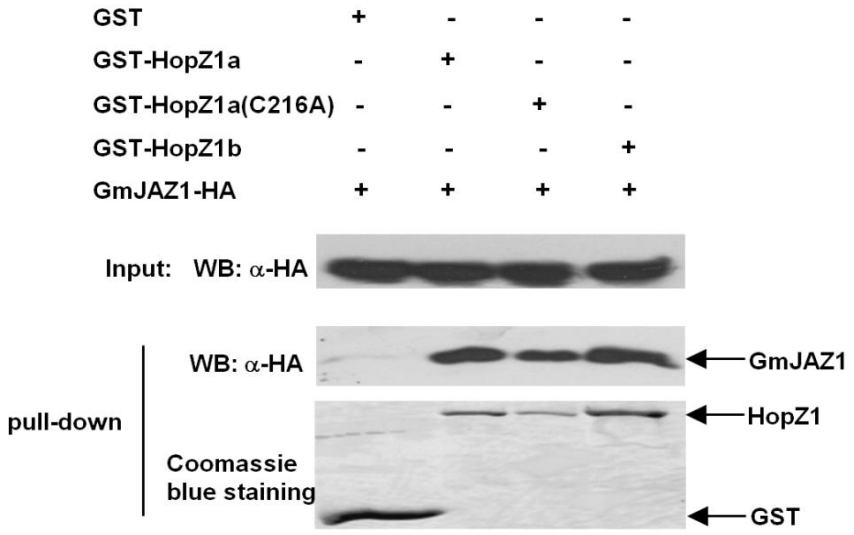


Figure 1.4. HopZ1 interacts with the soybean protein GmJAZ1.

HopZ1 and GmJAZ1 interact in vitro. GmJAZ1-HA and GST-HopZ1 proteins were expressed in *E. coli*. Co-precipitation of GmJAZ1 with HopZ1 was determined by western blots before (Input) and after affinity purification (Pull-down) using anti-HA antibody. The protein abundances of GST, GST-HopZ1a, GST-HopZ1b and GST-HopZ1a(C216A) on the affinity resins after washes were detected by Coomassie blue staining.

This experiment was repeated three times with similar results.



Previous studies reported that HopZ1 mainly locates on the plasma membrane with a sub-pool in the nucleus when HopZ1 is expressed in plant cells (Zhou et al, 2011). If GmJAZ1 physically interacts with HopZ1, they should co-localize. I examined the sub-cellular localization of GmJAZ1 in plant cells by expressing GmJAZ1-YFP in *N. 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. DAPI staining was used to visualize the nucleus in the cells. I observed fluorescence from both the plasma membrane and the nucleus (Figure 1.5). These results suggest that GmJAZ1 and HopZ1 could co-localize in these subcellular compartments of plant cells.

To further confirm that HopZ1a indeed enters the nucleus, Dr. Jian Yao at Michigan state University performed nuclear fractionation of *N. benthamiana* cells expressing HopZ1a(C216A). The catalytic mutant HopZ1a(C216A) was used in this experiment because the expression of wild-type HopZ1a triggers cell death in *N. benthamiana* (Ma et al, 2006; Zhou et al, 2009). Consistent with the previous confocal microscopy data (Zhou et al, 2011), the presence of HopZ1a(C216A) from both cytosolic and nuclear fractions were detected (Jiang et al, 2013). These data further confirmed that HopZ1a and GmJAZ1 could co-localize in both plasma membrane and nuclues.

I further used the bimolecular fluorescence complementation (BiFC) assay to determine the interaction site(s) of HopZ1a and GmJAZ1 in planta. HopZ1a(C216A), HopZ1b(C212A) and GmJAZ1 were fused to the nonfluorescent N-terminal domain of YFP (1-155aa, nYFP) and the C-terminal domain of YFP (156-239aa, 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 (Figure 1.6), which is 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.

Figure 1.5. Subcellular localization of GmJAZ1 in plant cells.

GmJAZ1-YFP was transiently expressed in *N. benthamiana* and the fluorescence was observed 48 hours post *Agro*-infiltration. DAPI was used to stain the nucleus. This experiment was repeated three times with similar results.

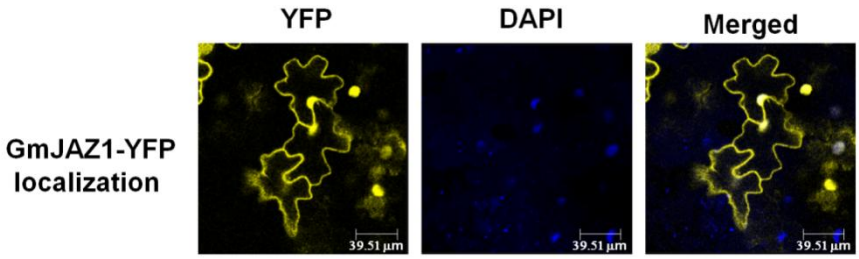
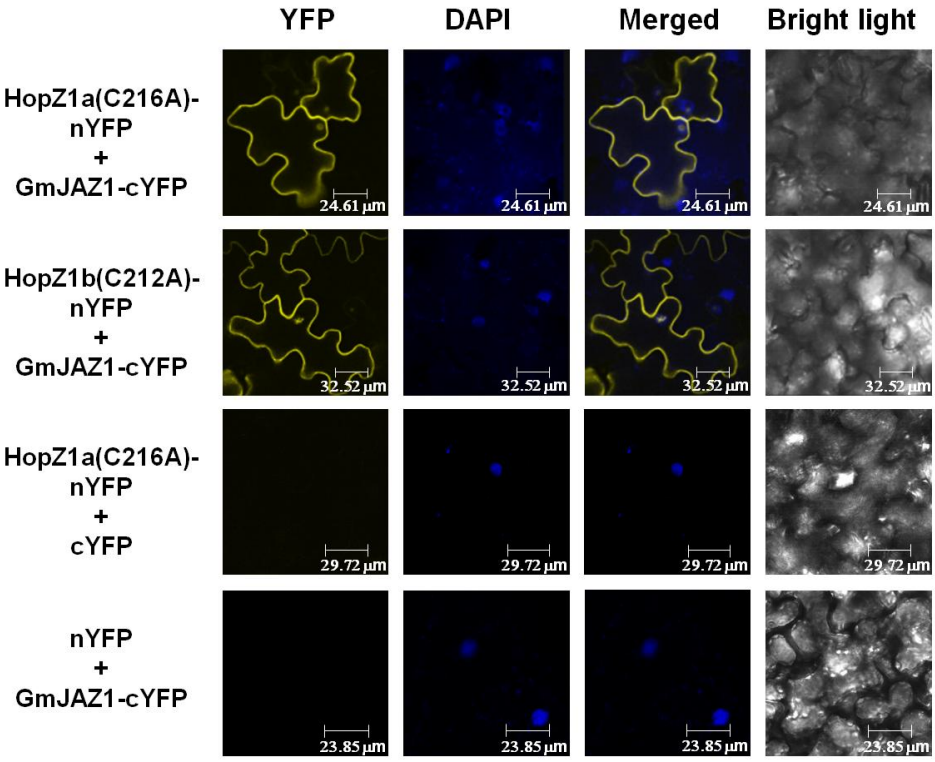


Figure 1.6. Bimolecular fluorescence complementation analysis of HopZ1-GmJAZ1 interaction in planta.

HopZ1a(C216A)-nYFP/HopZ1b(C212A)-nYFP and GmJAZ1-cYFP were co-expressed in *N. benthamiana* using *Agrobacterium*-mediated transient expression. Leaves co-infiltrated with *Agrobacterium* carrying GmJAZ1-cYFP + nYFP and cYFP + HopZ1a(C216A)-nYFP were used as negative controls. Fluorescence was detected by confocal microscopy from the infiltrated tissues 48 hours post inoculation. DAPI was used to stain the nuclei.

These experiments were repeated three times with similar results.



The Jas domain mediates the interaction between HopZ1 and GmJAZ1

JAZ proteins share three conserved domains: the C-terminal Jas motif (Yan et al, 2007), the ZIM domain in the central region (Vanholme et al, 2007), and a weakly conserved N-terminal region (Thines et al, 2007). I next investigated which domain(s) is responsible for the HopZ1-GmJAZ1 interaction. For this purpose, I constructed GmJAZ1 truncates with various regions of the protein deleted. These GmJAZ1 truncates were expressed in *E. coli* and tested for their interaction with HopZ1a and HopZ1b. My data suggest that the C-terminal Jas domain is the major contributor to JAZ interaction with HopZ1 (Figure 1.7). GmJAZ1 truncates 1 and 2 (T1 and T2), which have the Jas domain deleted, no longer interact with HopZ1; furthermore, GmJAZ1 truncate 3 (T3) and GmJAZ1ΔZIM are still able to interact with HopZ1, although they are deleted with the N-terminal domain or the ZIM domain, respectively. These data suggest that the Jas domain is required for GmJAZ1-HopZ1 interaction, whereas the ZIM domain and the N-terminal region are dispensable.

The central domain of HopZ1a is responsible for the interaction

We previously reported that HopZ1 interacts with GmHID1 mainly through the N-terminal domain of HopZ1 (Zhou et al, 2011). Although HopZ1 interacts with both GmHID1 and GmJAZ1, there is no obvious sequence similarity between these two proteins. Therefore, I characterized the specific region(s) in HopZ1a that is essential for its interaction with GmJAZ1. For this purpose, I constructed five HopZ1a truncates, expressed them in *E. coli*, and tested them for their interaction with GmJAZ1 using in vitro pull-down assays. The results showed that the central domain (63-244 aa) containing the catalytic triad is sufficient to mediate the interaction of

HopZ1a with GmJAZ1 (Figure 1.8). Interestingly, the construct T5 (63-369 aa) that contains the central and the C-terminal domain of HopZ1a did not interact with GmJAZ1 in this in vitro assay. We suspect that this HopZ1a truncate might not fold correctly or the C-terminal domain may somehow interfere with the interaction with GmJAZ1, which is mainly mediated through the central domain.

Figure 1.7. HopZ1 interacts with GmJAZ1 via the Jas domain.

In vitro pull-down assay was carried out using GST-HopZ1 and 6×HIS-tagged GmJAZ1 truncates. Full-length GmJAZ1 and maltose binding protein (MBP) were used as positive and negative controls, respectively. Co-precipitation of the truncated GmJAZ1 proteins with HopZ1 was detected by western blotting before (Input) and after affinity purification (Pull-down) using anti-HIS antibody.

These experiments were repeated three times with similar results.

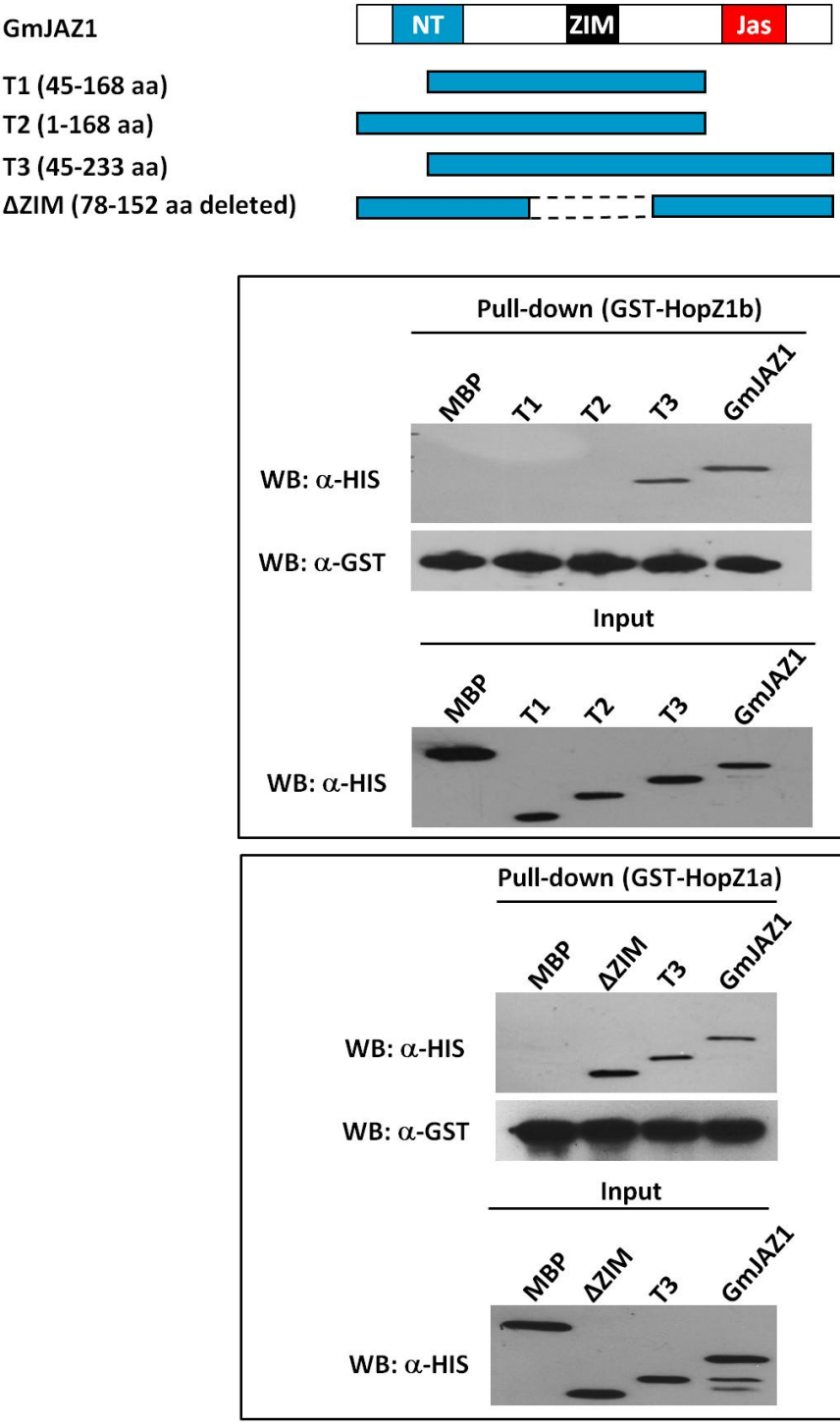
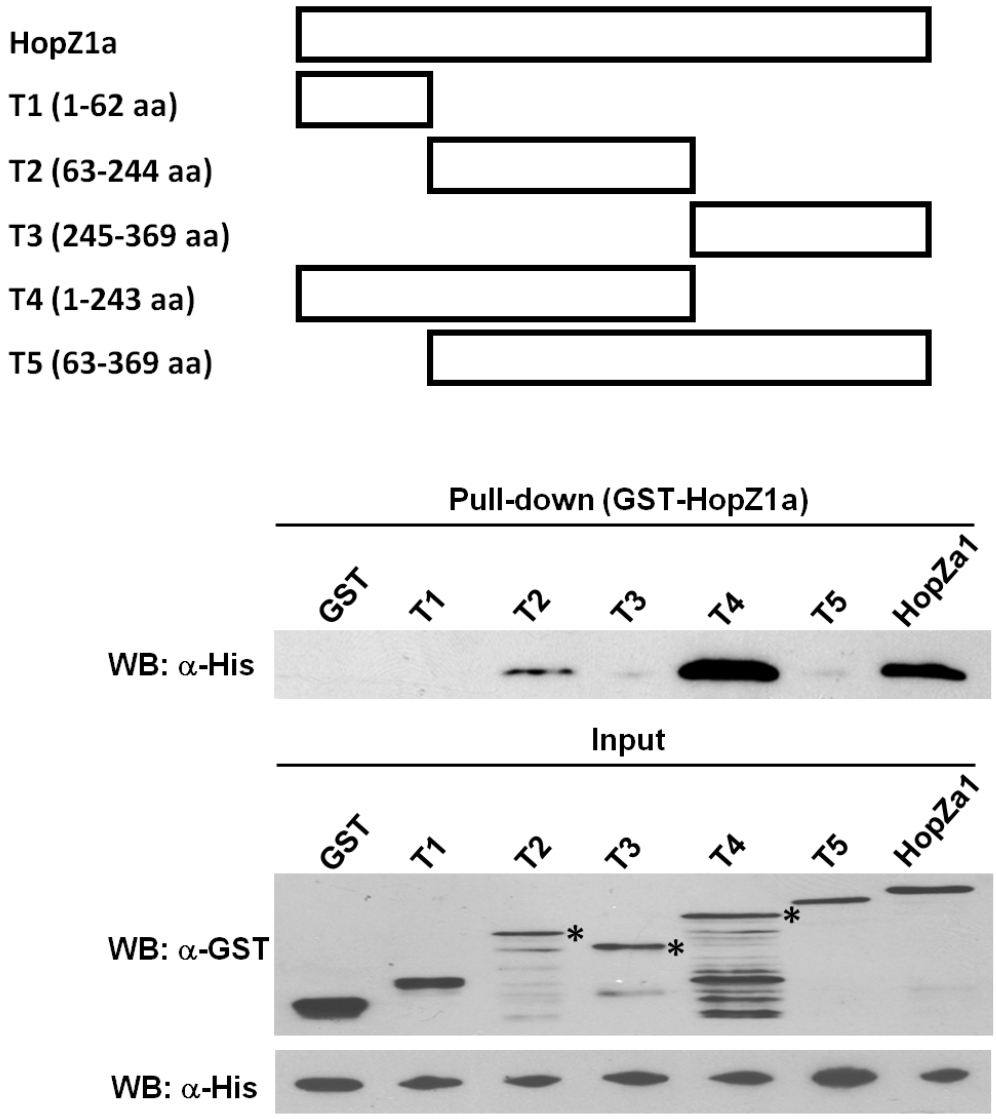

GmJAZ1
NT
ZIM
Jas
T1 (45-168 aa)
T2 (1-168 aa)
T3 (45-233 aa)
ΔZIM (78-152 aa deleted)
Pull-down (GST-HopZ1b)
MBP
T1
T2
T3
GmJAZ1
WB: α-HIS
WB: α-GST
Input
MBP
T1
T2
T3
GmJAZ1
WB: α-HIS
Pull-down (GST-HopZ1a)
MBP
ΔZIM
T3
GmJAZ1
WB: α-HIS
WB: α-GST
Input
MBP
ΔZIM
T3
GmJAZ1
WB: α-HIS

Figure 1.8. The central domain of HopZ1a is required and sufficient for interaction with GmJAZ1.

Five GST-tagged HopZ1a truncates were expressed in *E. coli* (T1: 1-62 aa; T2: 63-244 aa; T3: 245-369 aa; T4: 1-243 aa; T5: 63-369 aa). Precipitation of 6×HIS-GmJAZ1 proteins with HopZ1a truncates was detected by western blotting using anti-HIS antibody. Protein bands with the correct sizes are labeled with *.

These experiments were repeated twice with similar results.


HopZ1a
T1 (1-62 aa)
T2 (63-244 aa)
T3 (245-369 aa)
T4 (1-243 aa)
T5 (63-369 aa)
Pull-down (GST-HopZ1a)
GST
T1
T2
T3
T4
T5
HopZa1
WB: α-His
Input
GST
T1
T2
T3
T4
T5
HopZa1
WB: α-GST
WB: α-His

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* (Zhou et al, 2011). Therefore, I investigated whether HopZ1 can also induce the degradation of GmJAZ1.

GmJAZ1-FLAG and HopZ1-HA or HopZ1(C216A)-HA were co-expressed in *N. benthamiana*. The abundances of GmJAZ1 were determined at 20 hpi before the onset of visible cell death symptoms caused by HopZ1, which usually starts at 30 hpi. We chose 20 hpi because the expression level of GmJAZ1 was too low for protein abundance analysis at earlier time points.

A significant reduction of the GmJAZ1 protein level was observed in *N. benthamiana* leaves co-expressing wild-type HopZ1a-HA or HopZ1b-HA, compared to leaves expressing the HopZ1 catalytic mutants or infiltrated with *Agrobacterium* carrying the empty vector (Figure 1.9). These results suggest that, similar to GmHID1, both HopZ1a and HopZ1b induce the degradation of GmJAZ1 in plant cells and the degradation requires the enzymatic activity of HopZ1.

Incubation of GmJAZ1 and HopZ1a proteins purified from *E. coli* did not lead to observable changes in the abundance of GmJAZ1 in vitro (Figure 1.4). We suspected that a plant factor(s) might be required for this process. Therefore, I performed a semi-in vitro degradation assay by incubating proteins extracted from *N. benthamiana* tissues expressing GmJAZ1-FLAG or FLAG-HopZ1a individually. Total proteins were extracted from leaves transiently expressing GmJAZ1 or HopZ1a through *Agro*-infiltration. The protein samples were mixed and incubated at 4°C for six hours before the abundances of GmJAZ1 proteins were examined by western blots using anti-

FLAG antibodeis. Again, a significant decrease in GmJAZ1 protein level was observed in the presence of wild-type HopZ1a, but not the catalytic mutant HopZ1a(C216A) (Figure 1.10). These data further confirmed that HopZ1a induces GmJAZ1 degradation in plant cells.

Figure 1.9. HopZ1 induces the degradation of GmJAZ1 when the proteins were co-expressed in *N. benthamiana*.

GmJAZ1-FLAG and HopZ1-HA were transiently expressed in *N. benthamiana* and the abundance of GmJAZ1 was detected by western blots at 20 hpi. The same protein gel was stained with Coomassie blue to show equal loading.

This experiment was repeated three times with similar results.

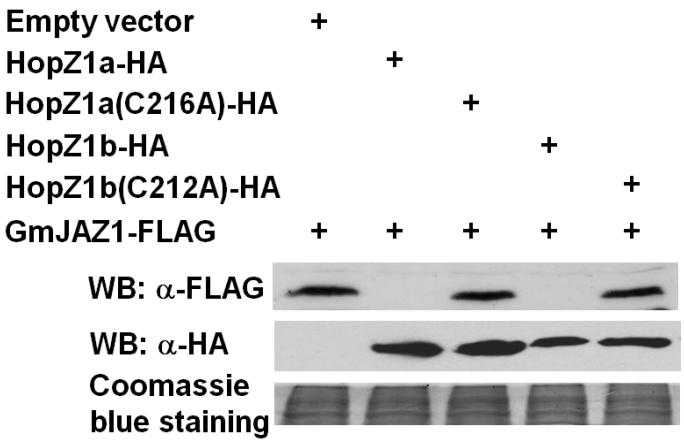
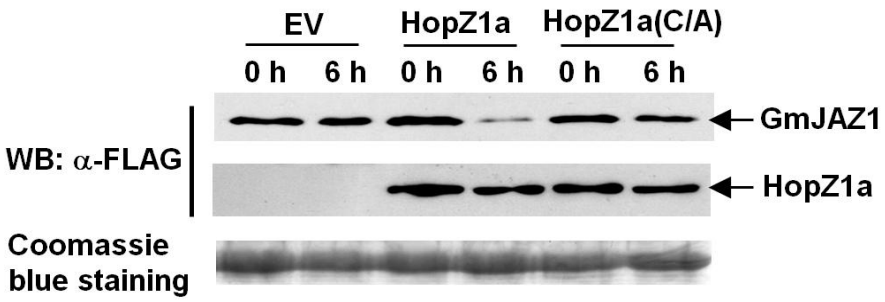


Figure 1.10. HopZ1a induces GmJAZ1 degradation using a semi-in vitro assay.

GmJAZ1-FLAG and 3×FLAG-HopZ1a or 3×FLAG-HopZ1a(C216A) were transiently expressed in *N. benthamiana* individually. Total proteins were extracted from the infiltrated leaves 20 hours post *Agro*-infiltration, mixed in equal volume, and incubated at 4°C for six hours. The abundances of GmJAZ1-FLAG were then analyzed by western blots.

This experiment was repeated three times with similar results.



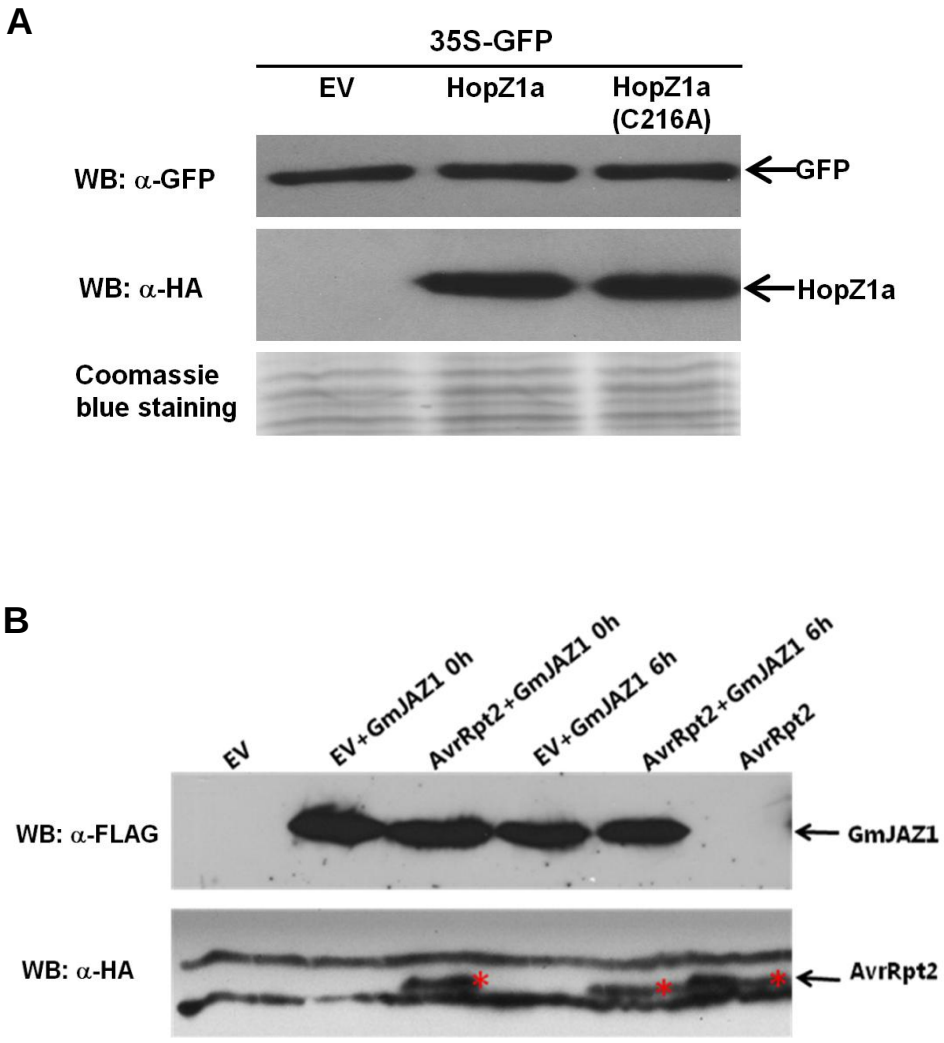
To exclude the possibility that the reduced GmJAZ1 protein levels might have been resulted from cell death triggered by wild-type HopZ1 in *N. benthamiana*, I conducted two control experiments. Firstly, I co-expressed the green fluorescence protein (GFP) with HopZ1a-HA or HopZ1a(C216A)-HA in *N. benthamiana*. The GFP protein levels remained unchanged in the presence of either wild-type or the catalytic mutant of HopZ1a (Figure 1.11A). Secondly, I performed the semi-in vitro degradation assay of GmJAZ1 in the presence of AvrRpt2, which is a *P. syringae* Type III effector that also elicits cell death in *N. benthamiana* (Mudgett & Staskawicz, 1999). Incubation with protein extracts expressing AvrRpt2 did not change the abundance of GmJAZ1 (Figure 1.11B). This suggests that the reduced abundance of GmJAZ1 was not a result of HopZ1-induced cell death in *N. benthamiana*. In summary, both experiments confirm specific GmJAZ1 degradation in plant cells by HopZ1a.

Figure 1.11. HopZ1a-triggered GmJAZ1 degradation in *N. benthamiana* is independent of plant cell death.

(A) GFP protein levels were 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.


A
35S-GFP
EV
HopZ1a
HopZ1a (C216A)
WB: α-GFP
GFP
WB: α-HA
HopZ1a
Coomassie blue staining
B
EV
EV+GmJAZ1 0h
AvrRpt2+GmJAZ1 0h
EV+GmJAZ1 6h
AvrRpt2+GmJAZ1 6h
AvrRpt2
WB: α-FLAG
GmJAZ1
WB: α-HA
AvrRpt2

HopZ1a interacts with Arabidopsis JAZs

Because GmJAZ1 is an ortholog of *Arabidopsis* JAZ proteins (AtJAZs), I examined whether HopZ1a also targets AtJAZs. This is important because direct targets of HopZ1a in *Arabidopsis* have not been identified.

Arabidopsis produces twelve JAZ orthologs (Figure 1.12). Phylogenetic analyses show the sequence similarity between GmJAZ1 and AtJAZs using full length protein sequences (Figure 1.12A). A similar phylogenetic relationship between GmJAZ1 and AtJAZs was also observed using sequences of the Jas domain (Figure 1.12B), which mediates the interaction of GmJAZ1 with HopZ1. I tested seven AtJAZs for their interactions with HopZ1a using in vitro pull-down. My data showed that AtJAZ2, AtJAZ5, AtJAZ6, AtJAZ8 and AtJAZ12 interacted with HopZ1a in vitro (Figure 1.13). Although AtJAZ1 shares the highest sequence similarity with GmJAZ1, the interaction of AtJAZ1 with HopZ1a could not be determined because I was unable to express AtJAZ1 in *E. coli* at a level suitable for the pull-down assay.

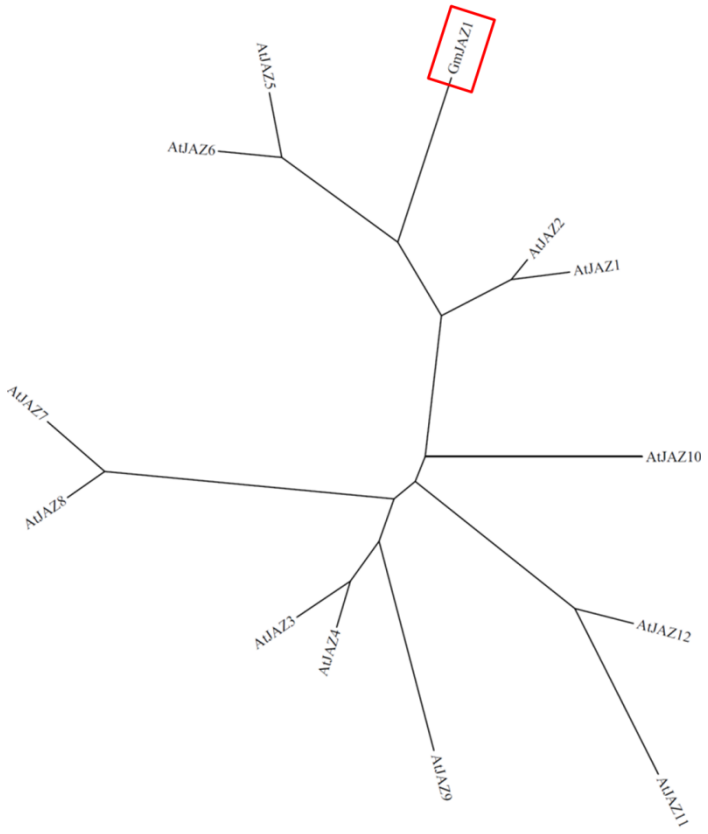
I next confirmed the interaction between HopZ1a and AtJAZ6 in planta using the BiFC assays. HopZ1a(C216A) and AtJAZ6 were cloned into vectors pSPYNE and pSPYCE respectively (Walter et al, 2004), and transiently expressed in *N. benthamiana* using *Agro*-infiltration. Protein-protein interaction events were examined at 48 hpi using confocal microscopy. Similar to HopZ1a-GmJAZ1 interaction, yellow fluorescence was observed from the plasma membrane and the nucleus in cells co-expressing HopZ1a(C216A)-nYFP and AtJAZ6-cYFP (Figure 1.14). AtJAZ6 by itself was mainly located in the nucleus (Figure 1.15). These data suggest that HopZ1a and AtJAZ6 co-localize and interact in plant cells.

Figure 1.12. Phylogenetic analyses of JAZs.

(A) Phylogenetic analysis of GmJAZ1 and AtJAZs. The PhyML tree was generated using full-length protein sequences by Seaview (Gouy et al, 2010).

(B) Phylogenetic analysis of GmJAZ1 and AtJAZs based on Jas domain sequences. Parsimony tree was generated using sequences right after the conserved ZIM domain. Sequences from the Jas domain only are too short to generate a phylogenetic tree with acceptable confidence.

A


GmJAZ1
AtJAZ5
AtJAZ6
AtJAZ2
AtJAZ1
AtJAZ7
AtJAZ10
AtJAZ8
AtJAZ3
AtJAZ4
AtJAZ12
AtJAZ9
AtJAZ11

B

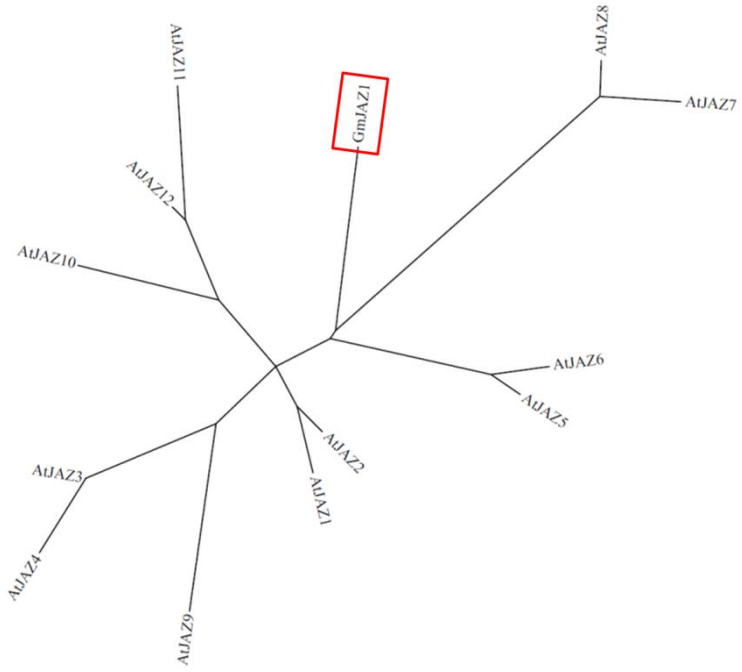

AtJAZ11
AtJAZ8
AtJAZ7
GmJAZ1
AtJAZ12
AtJAZ10
AtJAZ6
AtJAZ5
AtJAZ2
AtJAZ3
AtJAZ1
AtJAZ4
AtJAZ9

Figure 1.13. HopZ1a interacts with AtJAZs in vitro.

MBP-AtJAZ-HIS and GST-HopZ1a were expressed and purified from *E. coli*. Co-precipitation of MBP-AtJAZ-HIS with GST-HopZ1a was detected by western blots before (Input) and after affinity purification (Pull-down) using anti-HIS antibody. This experiment was repeated three times with similar results.

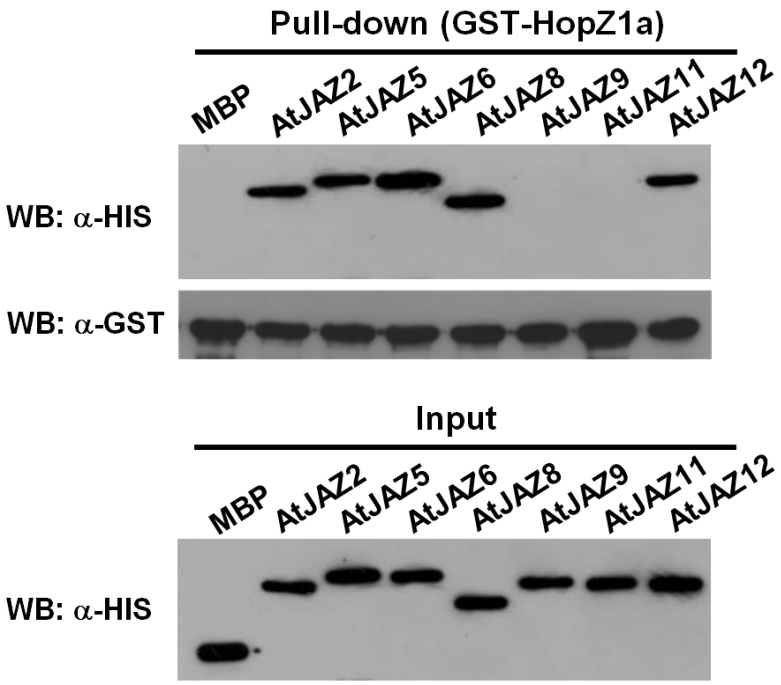


Figure 1.14. Bimolecular fluorescence complementation analysis showing the interaction between HopZ1a and AtJAZ6 in planta.

HopZ1a(C216A)-nYFP and AtJAZ6-cYFP were co-expressed in *N. benthamiana*. Fluorescence in the infiltrated leaves was monitored by confocal microscopy at 48 hours post *Agro*-infiltration. DAPI was used to stain the nuclei.

This experiment was repeated three times with similar results.

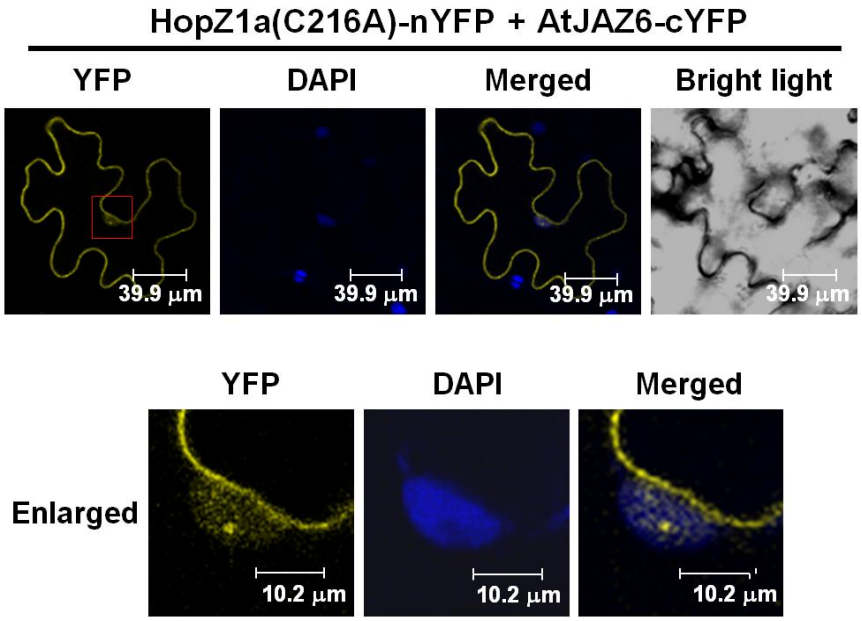
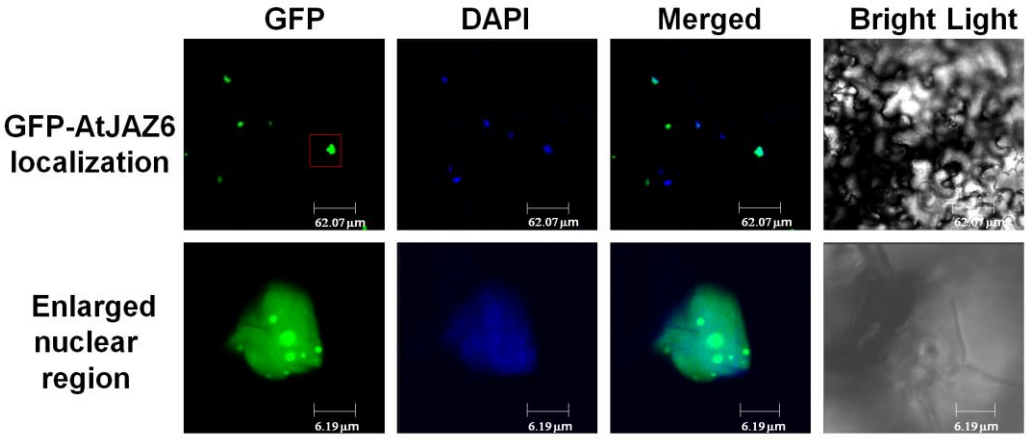


Figure 1.15. Subcellular localization of AtJAZ6 in plant cells.

GFP-AtJAZ6 was transiently expressed in *N. benthamiana* and the fluorescence was observed 48 hours post *Agro*-infiltration. DAPI was used to stain the nucleus. The boxed nuclear area is enlarged in the lower panels.

This experiment was repeated twice with similar results.



HopZ1a acetylates JAZs in vitro

Several effectors from the YopJ family, including HopZ1a, have been shown to possess the acetyltransferase activities. To determine whether JAZs are substrates of HopZ1a, I performed in vitro enzymatic assays using C14-labeled acetyl-CoA.

Recombinant HIS-SUMO-HopZ1a or HIS-SUMO-HopZ1a(C216A) proteins were expressed in *E. coli* and purified using nickel resins. The HIS-SUMO tag was then removed by ubiquitin like protease 1 (ULP1). Tag-free HopZ1a or HopZ1a(C216A) proteins were incubated with the purified HIS-GmJAZ1or MBP-AtJAZ6-HIS proteins in the presence of the cofactor inositol hexakisphosphate (IP6). The acetylations of HopZ1a, GmJAZ1 and AtJAZ6 were then detected by autoradiography as previously described (Mittal et al, 2010).

My experiments showed that both GmJAZ1 (Figure 1.16A) and AtJAZ6 (Figure 1.16B) can be 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 in part due to the low expression level of GmJAZ1 in *E. coli*, which only allowed me to use a much lower amount (1 μg), compared to AtJAZ6 (10 μg) in the reactions. Nonetheless, I 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.

A background level of acetylation in the tagged AtJAZ6 (MBP-AtJAZ6-HIS) was observed when it was incubated with HopZ1a(C216A). Although this background acetylation was very weak compared to the acetylation of MBP-AtJAZ6-HIS by wild-type HopZ1a, I decided to use the tag-free AtJAZ6 proteins to unambiguously confirm its acetylation by HopZ1a. In this experiment, I observed strong acetylation

of AtJAZ6 by HopZ1a, but not by HopZ1a(C216A) using 5 μg of AtJAZ6 in the reactions (Figure 1.16C). These results demonstrate that GmJAZ1 and AtJAZ6 are acetylation substrates of HopZ1a.

Figure 1.16. JAZs are acetylation substrates of HopZ1a.

(A) HopZ1a acetylates GmJAZ1 in vitro. Tag-free HopZ1a and HopZ1a(C216A), and HIS-GmJAZ1 were purified from *E. coli* and subjected to in vitro acetylation assays. The acetylated proteins were detected by autoradiography after exposure at -80°C for five days.

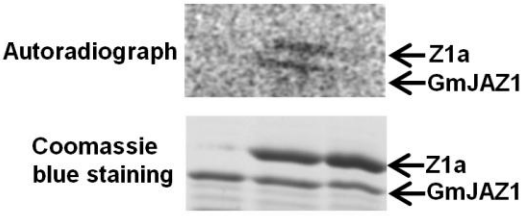
(B) HopZ1a acetylates MBP-AtJAZ6-HIS in vitro. Purified HopZ1a or HopZ1a(C216A) was incubated with MBP-AtJAZ6-HIS in the presence of C14-labeled acetyl-CoA and IP6.

(C) HopZ1a strongly acetylates the tag-free AtJAZ6. Tag-free HopZ1a or HopZ1a(C216A), and AtJAZ6 were purified from *E. coli* and subjected to in vitro acetylation assay.

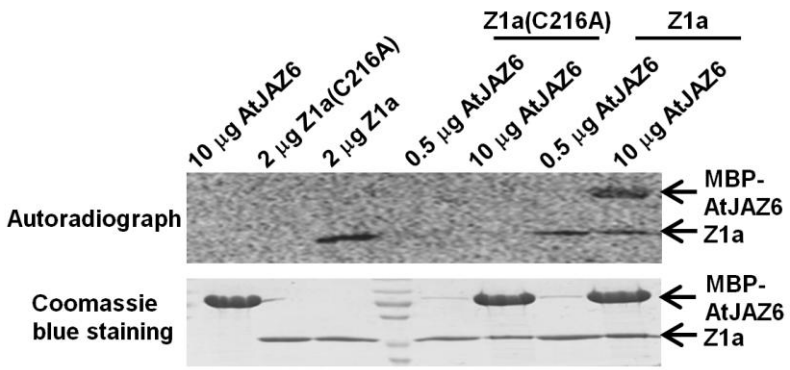
These experiments were repeated at least three times with similar results.

A

HopZ1a		+	
HopZ1a(C216A)			+
HIS-GmJAZ1 (1 μg)	+	+	+

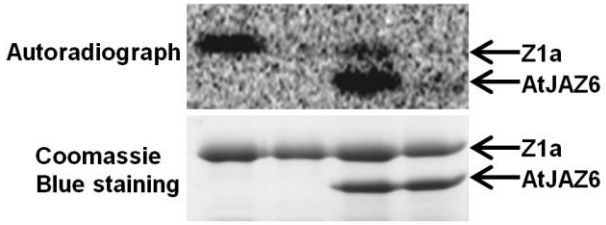


B



C

HopZ1a	+		+	
HopZ1a(C/A)		+		+
AtJAZ6			+	+



The Jas domain of AtJAZ6 is required for HopZ1a interaction

My previous experiments suggest that the conserved Jas domain is important for GmJAZ1 to bind HopZ1a. Therefore, I next examined the impact of the Jas domain in the interaction between AtJAZ6 and HopZ1a using a mutant AtJAZ6ΔJas, which was constructed by deleting ten highly conserved amino acids (from ser^{191} to lys^{200}) within the Jas domain of AtJAZ6. In vitro pull-down assay showed that unlike AtJAZ6, AtJAZ6ΔJas did not co-precipitate with HopZ1a (Figure 1.17A). Furthermore, AtJAZ6ΔJas was no longer acetylated by HopZ1a in vitro (Figure 1.17B). Importantly, when co-expressed with HopZ1a in *N. benthamiana*, AtJAZ6ΔJas was also no longer degraded by HopZ1a (Figure 1.17C). These results demonstrate that HopZ1a-induced AtJAZ6 degradation requires direct interaction with HopZ1a, and possibly acetylation by HopZ1a, which is mediated by the Jas domain.

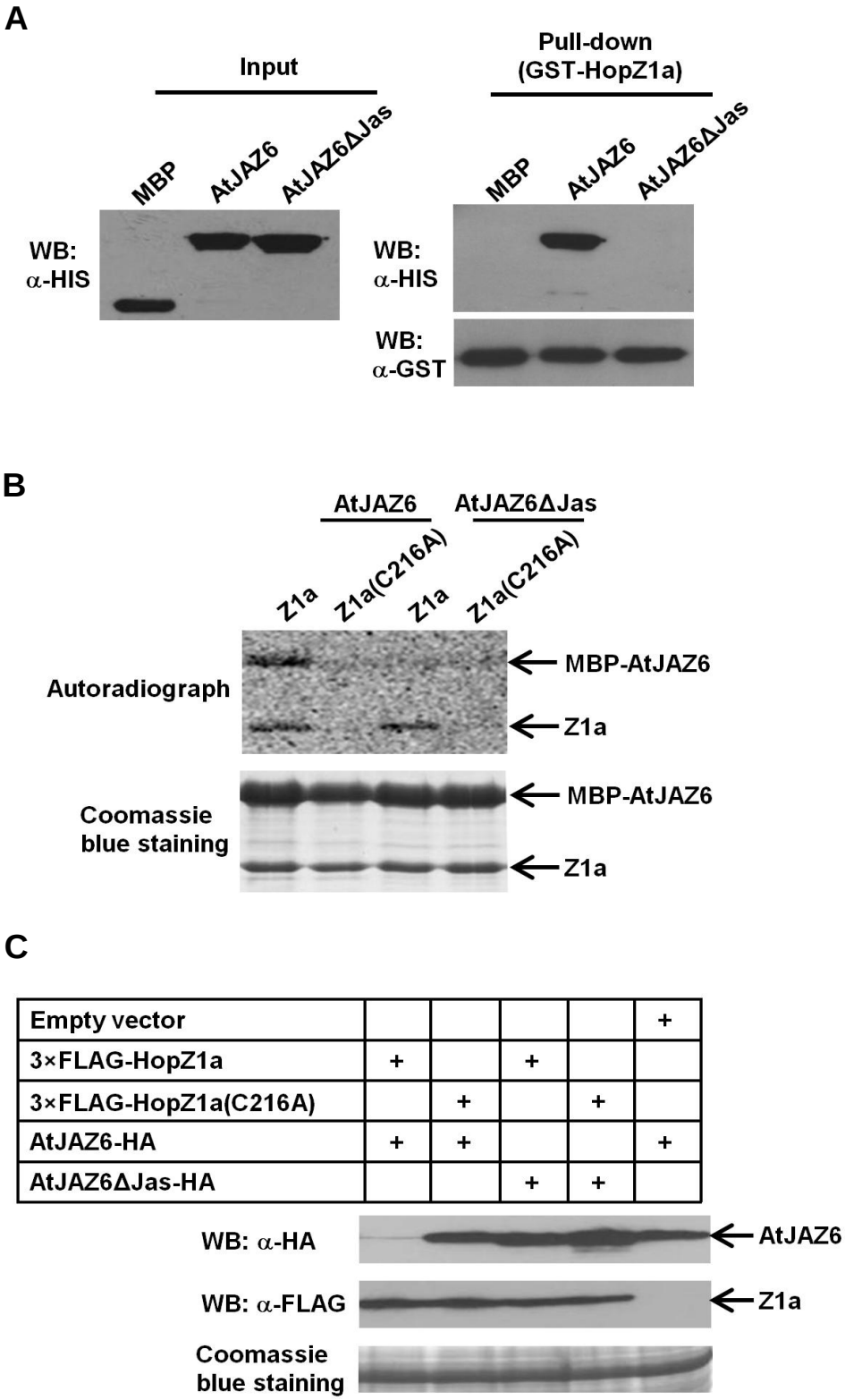
Figure 1.17. HopZ1a-induced JAZ degradation requires direct interaction of HopZ1a with AtJAZ6 via the Jas domain.

(A) The mutant AtJAZ6ΔJas no longer interacts with HopZ1a in vitro.

(B) HopZ1a no longer acetylates AtJAZ6ΔJas in vitro. This experiment was repeated at least three times with similar results.

(C) HopZ1a does not trigger the degradation of AtJAZ6ΔJas. AtJAZ6ΔJas-YFP-HA and 3×FLAG-HopZ1a were co-expressed in *N. benthamiana*. The abundances of AtJAZ6ΔJas were detected by western blots.

The in vitro pull-down and degradation experiments were repeated twice with similar results.


A
Input
Pull-down
(GST-HopZ1a)
MBP
AtJAZ6
AtJAZ6ΔJas
WB:
α-HIS
WB:
α-GST
B
AtJAZ6
AtJAZ6ΔJas
Z1a
Z1a(C216A)
Autoradiograph
MBP-AtJAZ6
Coomassie
blue staining
C
Empty vector
3×FLAG-HopZ1a
3×FLAG-HopZ1a(C216A)
AtJAZ6-HA
AtJAZ6ΔJas-HA
WB: α-HA
WB: α-FLAG
Coomassie
blue staining

HopZ1a triggers the degradation of AtJAZ1 during bacterial infection

Although degradation of GmJAZ1 and AtJAZ6 was observed when they were co-expressed with HopZ1a in *N. benthamiana*, it is important to determine whether HopZ1a can promote JAZ degradation during bacterial infection. For this purpose, transgenic *Arabidopsis* plants expressing *35S-HA-AtJAZ1* were inoculated with *P. syringae* producing HopZ1a or HopZ1a(C216A) (Jiang et al, 2013). The *Arabidopsis* pathogen *Pseudomonas syringae* pv. *tomato* strain DC3000 (*Pto*DC3000) is well-known to induce AtJAZ degradation through the production of coronatine, which acts as a JA-Ile mimic (Katsir et al, 2008). Western blots showed strong degradation of AtJAZ1 in *Arabidopsis* leaves infected with *Pto*DC3000. On the contrary, a mutant of *Pto*DC3000, *Pto*DC3118, which is deficient in coronatine production, can no longer degrade AtJAZs. Importantly, *Pto*DC3118 expressing HopZ1a from its native promoter also significantly reduced the abundance of AtJAZ1 at 6 hpi. Furthermore, the levels of AtJAZ1 remained unchanged in tissues inoculated with *Pto*DC3118 carrying the empty vector or expressing the catalytic mutant HopZ1a(C216A) (Jiang et al, 2013). These data strongly suggest that HopZ1a, injected into plant cells through the type III secretion system, can induce AtJAZ1 degradation during infection.

Decreased protein abundance by HopZ1a was also observed for AtJAZ2 and AtJAZ12, using *35S-HA-AtJAZ2* and *35S-HA-AtJAZ12* transgenic *Arabidopsis* plants (data not shown, performed by Dr. Jian Yao at Michigan State University). These results suggest that HopZ1a degrades multiple AtJAZs during bacterial infection.

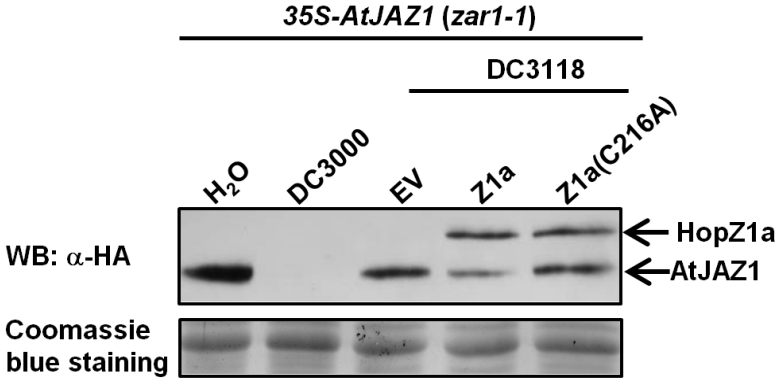
Because HopZ1a elicits HR in *Arabidopsis* ecotype Col-0, two experiments were performed by Dr. Jian Yao and me to exclude the possibility that HopZ1a-triggered AtJAZ1 degradation was indirectly resulted from the plant cell death process.

First, Dr. Jian Yao examined whether AvrRpt2 could also induce AtJAZ1 degradation. Although AvrRpt2 triggers HR in *Arabidopsis* Col-0, the abundance of AtJAZ1 was unchanged when the *HA-AtJAZ1*-expressing plants were inoculated with *Pto*DC3118 expressing AvrRpt2 (Jiang et al, 2013). Next, I generated the transgenic *Arabidopsis* line expressing *35S-HA-AtJAZ1* in the *zar1-1* mutant background, which is abrogated in HopZ1a-triggered HR (Lewis et al, 2010). Again, the AtJAZ1 protein level was significantly reduced by *Pto*DC3118 expressing HopZ1a but not *Pto*DC3118 expressing HopZ1a(C216A) (Figure 1.18), confirming that HopZ1a delivered by *P. syringae* leads to AtJAZ1 degradation in a cell death independent manner.

Figure 1.18. HopZ1a promotes the degradation of AtJAZ1 in *Arabidopsis*.

Six week-old *35S-HA-AtJAZ1 zar1-1 Arabidopsis* plants were inoculated with *Pto*DC3000, *Pto*DC3118 carrying the empty pUCP18 vector (EV), or *Pto*DC3118 expressing HopZ1a or HopZ1a(C216A). The protein abundance of AtJAZ1 was determined by western blot using anti-HA antibody. The protein gels were stained with Coomassie blue as loading controls.

This experiment was repeated three times with similar results.



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

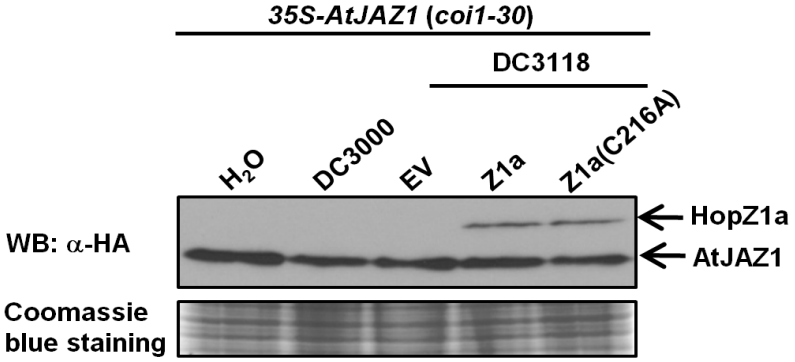
A major regulatory mechanism of JAZs in the presence of JA or coronatine is through COI-dependent ubiquitin-proteasome degradation. COI1 is an F-box protein that determines the substrate specificity of a Skp/Cullin/F-box (SCF) E3 ubiquitin ligase-SCF^{COI1} (Xie et al, 1998). Together with JAZs, COI1 is a critical component of the JA co-receptor complex (Katsir et al, 2008; Sheard et al, 2010; Thines et al, 2007). I examined whether COI1 is required for HopZ1a-induced JAZ degradation using the transgenic *Arabidopsis* line expressing *35S-HA-AtJAZ1* in the *coi1-30* (SALK_035548) mutant background (Seeds provided by Dr. Jian Yao at Michigan State University).

As expected, *Pto*DC3000, which induces JAZ degradation through coronatine production, was unable to reduce the abundance of AtJAZ1 in the absent of COI1. Interestingly, *Pto*DC3118 expressing HopZ1a also no longer induced the degradation of AtJAZ1 in the *coi1* mutant plants (Figure 1.19). These data suggest that, similar to coronatine- and JA-mediated AtJAZ degradation, COI1 is required for the degradation of AtJAZ1 by HopZ1a.

Figure 1.19. HopZ1a-triggered JAZ degradation is COI1-dependant.

HopZ1a-mediated degradation of AtJAZ1 is dependent on COI1. *35S-HA-AtJAZ1*, *coi1-30 Arabidopsis* plants were inoculated with *Pto*DC3000, *Pto*DC3118 carrying the empty pUC18 vector (EV), or *Pto*DC3118 expressing HopZ1a or HopZ1a(C216A). 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.



HopZ1a activates JA signaling

In *Arabidopsis*, JAZ proteins are repressors of JA transcription factors (e.g. AtMYC2) that activate JA-responsive genes (Boter et al, 2004; Lorenzo et al, 2004; Niu et al, 2011). Since HopZ1a induces the degradation of AtJAZ1, I examined whether it could activate JA signaling and induce the expression of JA-responsive genes during bacterial infection.

Real-time RT-PCR was carried out to determine the transcript levels of JA-responsive genes in *Arabidopsis*. Five-week old *zar1-1* plants were inoculated with *Pto*DC3118 expressing HopZ1a or HopZ1a(C216A) at OD_{600} = 0.2. The transcript levels of two early JA-responsive genes, *AtJAZ9* and *AtJAZ10* (Niu et al, 2011), were analyzed at 6 hpi. Both genes were induced by approximately ten fold in plants infected by *Pto*DC3118 carrying HopZ1a, whereas their expression was not changed in tissues infected by *Pto*DC3118 expressing HopZ1a(C216A) (Figure 1.20A). 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 *Pto*DC3000. 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 (Zheng et al, 2012). Because SA-associated defense confers resistance against biotrophic and hemibiotrophic pathogens, reduced SA accumulation would lead to enhanced susceptibility. Since coronatine is able to repress the expression of the SA synthetic enzyme isochorismate synthase gene 1 (*AtICS1*) in *Arabidopsis*, I therefore examined

the impact of HopZ1a on the expression of *AtICS1*. *Arabidopsis zar1-1* plants were inoculated with *Pto*DC3000 or *Pto*DC3118 carrying empty vector, HopZ1a, or HopZ1a(C216A). Consistent with the prior findings, the transcript abundance of *AtICS1* was reduced in plants infected with *Pto*DC3000 when compared to *Pto*DC3118 carrying the empty vector or HopZ1a(C216A) (Figure 1.20B). The expression of *AtICS1* was also significantly reduced in plants inoculated with *Pto*DC3118 expressing HopZ1a, to a similar level as that in leaves inoculated with *Pto*DC3000. These data confirmed that, like coronatine, HopZ1a activates JA signaling and represses SA accumulation during bacterial infection.

Figure 1.20. HopZ1a activates JA signaling and represses SA accumulation during bacterial infection in *Arabidopsis*.

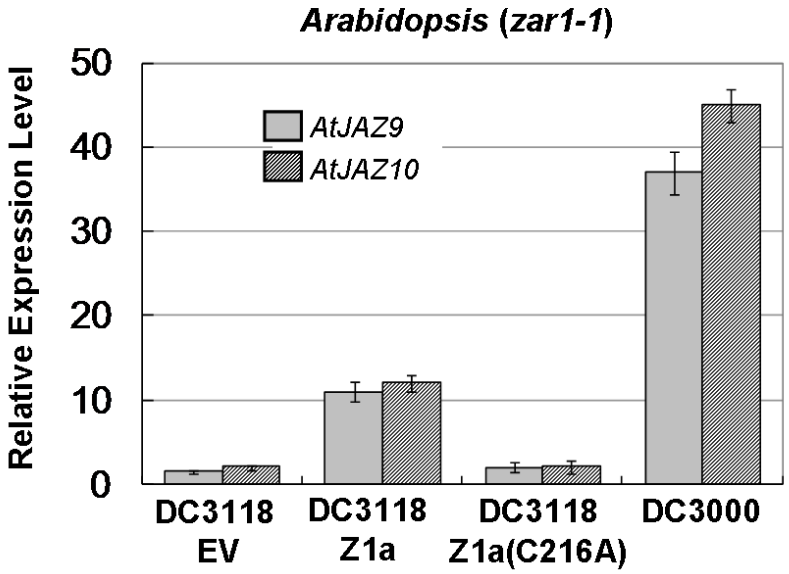
Arabidopsis zar1-1 mutant plants were inoculated with *Pto*DC3000 or *Pto*DC3118 carrying the empty pUC18 vector (EV), HopZ1a or HopZ1a(C216A). The transcript levels of the JA-responsive genes *AtJAZ9* and *AtJAZ10*, as well as the SA biosynthetic gene *AtICS1* were determined by quantitative RT-PCR.

(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 $MgSO_4$) samples. Values are means $\pm$ standard deviations (as error bars) (n=5).

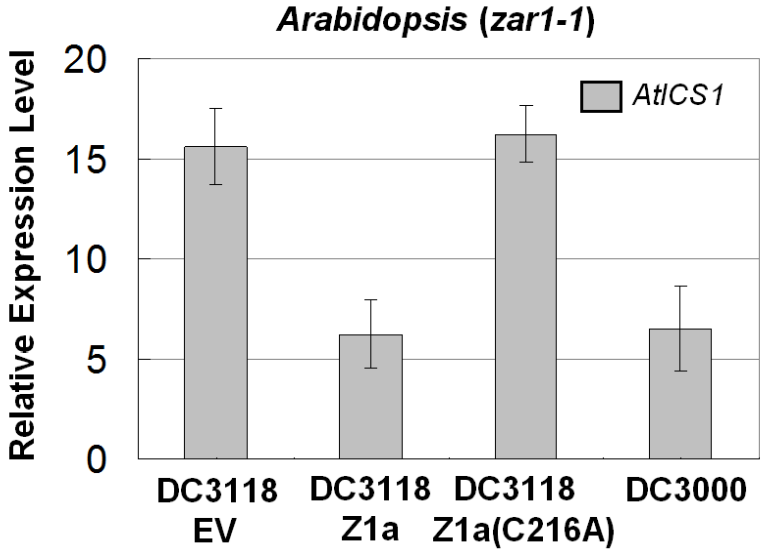
(B) HopZ1a reduces the expression of *AtICS1* in *Arabidopsis*. *AtICS1* transcript level was analyzed at 9 hpi using *AtUBQ5* as the internal standard. Values are means $\pm$ standard deviations (as error bars) (n=5).

All experiments were repeated at least five times with similar results.

A


Arabidopsis (zar1-1)
Relative Expression Level
50
40
30
20
10
0
AtJAZ9
AtJAZ10
DC3118 EV
DC3118 Z1a
DC3118 Z1a(C216A)
DC3000

B


Arabidopsis (zar1-1)
Relative Expression Level
20
15
10
5
0
AtICS1
DC3118 EV
DC3118 Z1a
DC3118 Z1a(C216A)
DC3000

COI1 is required for HopZ1a to activate JA signaling

Because COI1 is required for HopZ1a-induced degradation of AtJAZ1, we then examined whether COI1 is also required for HopZ1a to activate JA signaling in *Arabidopsis*. For this purpose, my colleague Ka-Wai Ma generated *coi1-1*, *zar1-1* double mutant *Arabidopsis* plants, which were inoculated with *Pto*DC3118 carrying the empty vector, HopZ1a or HopZ1a(C216A). I then 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 *Pto*DC3000, *Pto*DC3118 expressing HopZ1a was also unable to induce the expression of *AtJAZ9* and *AtJAZ10* (Figure 1.21A) or suppress the expression of *AtICS1* (Figure 1.21B). These data suggest that both the phytotoxin coronatine and the effector HopZ1a activate JA signaling in a COI1-dependant manner.

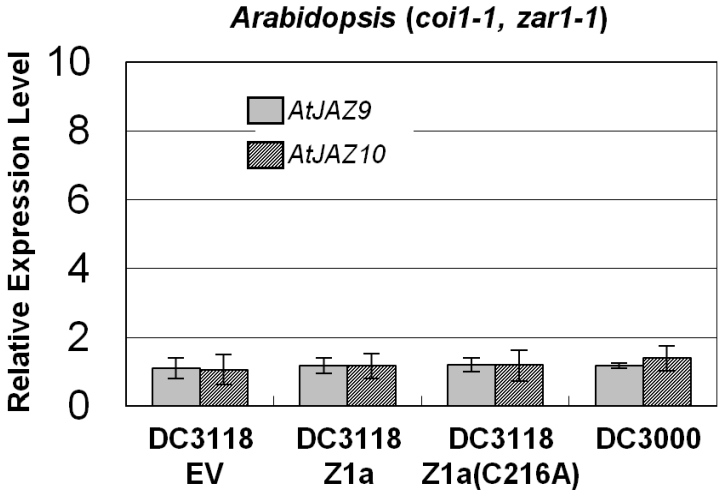
Figure 1.21. HopZ1a no longer activates JA signaling in *coi1-1* mutant *Arabidopsis*.

Arabidopsis coi1-1, zar1-1 mutant plants were inoculated with *Pto*DC3000 or *Pto*DC3118 carrying the empty pUC18 vector (EV), HopZ1a or HopZ1a(C216A). Relative expression was determined by comparing the normalized *AtJAZ9*, *AtJAZ10* or *AtICS1* transcript levels between the infected and the mock-inoculated samples (leaves infiltrated with 10mM $MgSO_4$). *AtUBQ5* was used as the internal standard. **(A)** Transcript abundances of the JA-responsive genes *AtJAZ9* and *AtJAZ10* were determined at 6 hpi.

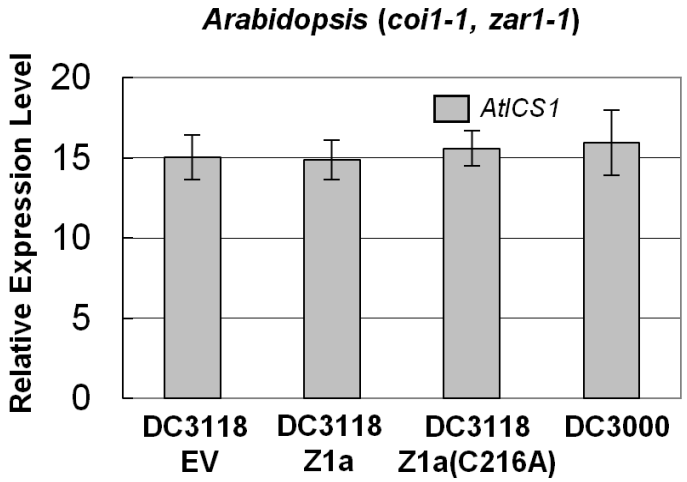
(B) Transcript level of *AtICS1* was determined at 9 hpi. Values are means $\pm$ standard deviations (as error bars) (n=3).

All experiments were repeated twice with similar results.

A


Arabidopsis (coi1-1, zar1-1)
Relative Expression Level
10
8
6
4
2
0
AtJAZ9
AtJAZ10
DC3118 EV
DC3118 Z1a
DC3118 Z1a(C216A)
DC3000

B


Arabidopsis (coi1-1, zar1-1)
Relative Expression Level
20
15
10
5
0
AtICS1
DC3118 EV
DC3118 Z1a
DC3118 Z1a(C216A)
DC3000

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

Coronatine facilitates the infection of *Pto*DC3000 by activating JA signaling in *Arabidopsis* (Brooks et al, 2005). The coronatine-deficient mutant *Pto*DC3118 exhibits a significant reduction in bacterial population especially when the plants are inoculated by dipping (Melotto et al, 2006). Since HopZ1a also induces the degradation of JAZs and activates JA signaling, I examined whether HopZ1a could complement the growth deficiency of *Pto*DC3118.

The *Arabidopsis zar1-1* mutant plants were dipping-inoculated by *Pto*DC3000 or *Pto*DC3118 carrying the empty vector, HopZ1a, or HopZ1a(C216A). Three days post infection (dpi), the bacterial populations of *Pto*DC3118 carrying the empty vector or expressing HopZ1a(C216A) were approximately 200 fold lower than that of *Pto*DC3000 (Figure 1.22A). Consistently, plants infected with *Pto*DC3000 displayed severe disease symptoms, whereas those infected with *Pto*DC3118 or *Pto*DC3118 expressing HopZ1a(C216A) looked rather healthy (Figure 1.22B). Importantly, *Pto*DC3118 expressing wild-type HopZ1a multiplied to a significantly higher level (about 10 fold) than *Pto*DC3118 or *Pto*DC3118 expressing HopZ1a(C216A) (Figure 1.22A). Plants infected with *Pto*DC3118(HopZ1a) also exhibited enhanced disease symptoms than *Pto*DC3118 (Figure 1.22B). Although the population of *Pto*DC3118(HopZ1a) is lower than that of *Pto*DC3000, this partial complementation of the growth deficiency of *Pto*DC3118 is consistent with the partial degradation of AtJAZ1 (Figure 1.18) and the lower levels of JA-responsive gene induction (Figure 1.20A) by *Pto*DC3118(HopZ1a) compared to *Pto*DC3000.

To further confirm that the function of HopZ1a is specifically related to its ability to activate the JA pathway, I introduced HopZ1a into *Pto*DC3000 and performed the same bacterial growth assay. HopZ1a was previously shown to enhance the infection of *Pto*DC3000 (Lewis et al, 2010). However, despite numerous trials, I did not observe any enhancement of HopZ1a on in planta multiplication of *Pto*DC3000. In fact, a decrease in the population of *Pto*DC3000(HopZ1a) was consistently detected compared to *Pto*DC3000 carrying the empty vector (Figure 1.22). Thus, HopZ1a can partially substitute for coronatine to promote bacterial infection.

Because COI1 is required for HopZ1a to activate JA signaling, my colleague Ka-Wai Ma then examined whether COI1 is also required for the virulence activity of HopZ1a in *Arabidopsis*. *coi1-1*, *zar1-1* double mutant *Arabidopsis* plants were inoculated by *Pto*DC3118 carrying the empty vector, or expressing HopZ1a or HopZ1a(C216A). The bacterial populations of these three strains remained the same (Jiang et al, 2013), confirming that the virulence activity of HopZ1a requires COI1.

The potential myristoylation site of HopZ1a is not required for its virulence function

HopZ1a contains a potential N-terminal myristoylation site (Gly2) which was proposed to direct the association of HopZ1a with plasma membrane in plant cells (Lewis et al, 2008; Zhou et al, 2009). The mutant HopZ1a(G2A) with Gly2 replaced with an alanine was mostly present in the soluble faction but not the membrane fraction when expressing in *N. benthamiana* cells (Lewis et al, 2008). Besides, HopZ1a(G2A)-YFP fusion proteins mainly located in the cytoplasm and nucleus in the onion epidermal cells under confocal microscope detection (Zhou et al, 2009).

This Gly2 site was also required for HopZ1a-triggered hypersensitive response (HR) in both *Arabidopsis* and soybean (Lewis et al, 2008; Zhou et al, 2009). However, it is unclear that whether this site is important for the virulence function of HopZ1a. As HopZ1a interacts with and degrades JAZ transcription repressors, which are believed to function in the nucleus, I therefore examined whether the plasma membrane localization of HopZ1a is required for the virulence function of HopZ1a using the HopZ1a(G2A) mutant.

For this purpose, I determined the bacterial population of *Pto*DC3118 carrying HopZ1a(G2A) in *Arabidopsis zar1-1* mutant plants at 3 dpi. *Pto*DC3118 carrying HopZ1a(G2A) grew to the similar level as *Pto*D3118 carrying wild-type HopZ1a in *zar1-1* plants (Figure 1.23). This result demonstrates that HopZ1a(G2A) retained the virulence activity to promote *Pto*D3118 multiplication in *Arabidopsis*, indicating that the potential myristoylation site or in other word, the plasma membrane localization, is not required for the virulence function of HopZ1a.

Taken together, our experiments showed that HopZ1a enhanced *P. syringae* infection in *Arabidopsis* in a manner similar to coronatine, which is a potent activator of JA signaling during bacterial infection.

Figure 1.22. HopZ1a partially complements the virulence function of coronatine during bacterial infection.

(A) HopZ1a promotes the multiplication of *Pto*DC3118 in *Arabidopsis*. *Arabidopsis zar1-1* plants were dip-inoculated with *Pto*DC3000 carrying pUCP20tk (EV), pUCP20tk::*hopZ1a-HA*, or *Pto*DC3118 carrying pUCP18 (EV), pUCP18::*hopZ1a-HA* or pUCP18::*hopZ1a(C216A)-HA* at OD_{600}=0.2 (approximately 2×10^8cfu/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) Photos of the infected plants showing disease symptoms were taken at 3 dpi This experiment was repeated at least three times with similar results.

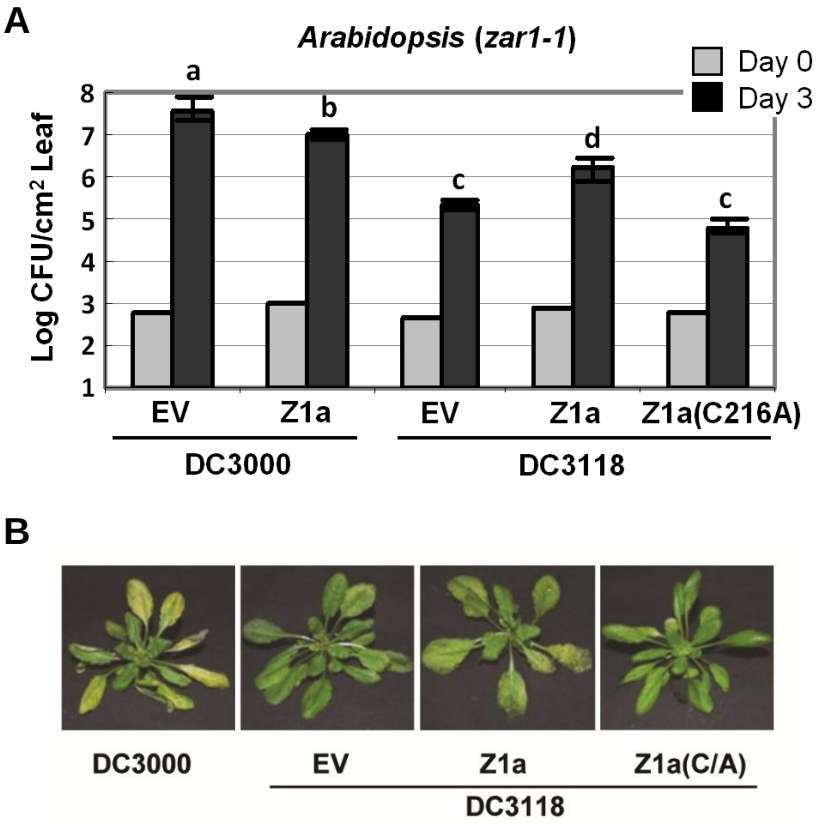
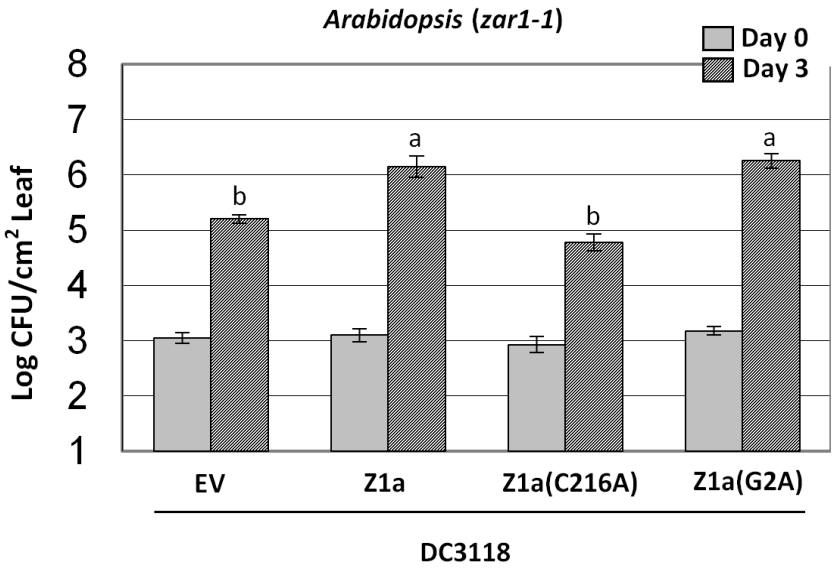

A
Arabidopsis (zar1-1)
Day 0
Day 3
Log CFU/cm2 Leaf
8
7
6
5
4
3
2
1
a
b
c
d
c
EV
Z1a
EV
Z1a
Z1a(C216A)
DC3000
DC3118
B
DC3000
EV
Z1a
Z1a(C/A)
DC3118

Figure 1.23. The mutant HopZ1a(G2A) retains the ability to promote *Pto*DC3118 infection to the same extent as wild-type HopZ1a.

*Pto*DC3118 expressing the empty vector (EV), HopZ1a, HopZ1a(C216A) or HopZ1a(G2A) were used to dip-inoculate five-week old *Arabidopsis zar1-1* plants. Colony forming units (cfu) were determined at 0 day and 3 dpi. The average colony forming units per square centimeter (cfu/cm^2) 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 (two tailed t-test p<0.01).

This experiment was repeated twice with similar results.



HopZ1b does not have autoacetylation activity

The two functional alleles of HopZ1, HopZ1a and HopZ1b share 72% identity and 81% similarity in their full length amino acids. As HopZ1a exhibits a strong autoacetylation activity and evidently acetylates GmJAZ1 and AtJAZ6, it is interesting to test whether HopZ1b has a similar enzymatic activity. Unfortunately, in several independent in vitro acetylation assays, HopZ1b showed no autoacetylation activity and it does not acetylate AtJAZ6 (Figure 1.24). This suggests that unlike HopZ1a, HopZ1b may not function as an acetyltransferase, at least under my in vitro experimental condition.

Back-to-back sequence comparison revealed two major indels in the amino acid sequences of HopZ1a and HopZ1b (Morgan et al, 2010). They are 28-31 aa of HopZ1a, and 251-253 aa of HopZ1b. The regions surrounding these two indels also show relatively higher sequence variability between the two HopZ1 alleles. We suspected that the sequence variability of these regions might be responsible for the differences in their enzymatic activities. To test this hypothesis, I examined the influence of the second indel on the acetylation activity of HopZ1 using the chimeric proteins HopZ1a1b-2 (HopZ1a1–244 aa fused to HopZ1b240–368 aa) and HopZ1b1a-2 (HopZ1b1–239 aa fused to HopZ1a245–369 aa) (Morgan et al, 2010).

In vitro acetylation assays showed that HopZ1a1b-2 can be autoacetylated in a similar manner as wild type HopZ1a, and it can also strongly acetylate AtJAZ6. Interestingly, HopZ1b1a-2 exhibited autoacetylation activity, but it could not acetylate AtJAZ6. This indicates that the sequence differences in HopZ1a and HopZ1b are important for their enzymatic activities. Further mass spectrometry analyses as well as the following enzymatic activity examinations of site-directed

HopZ1a mutants are required to unravel the differences between HopZ1a and HopZ1b in terms of their enzymatic activities.

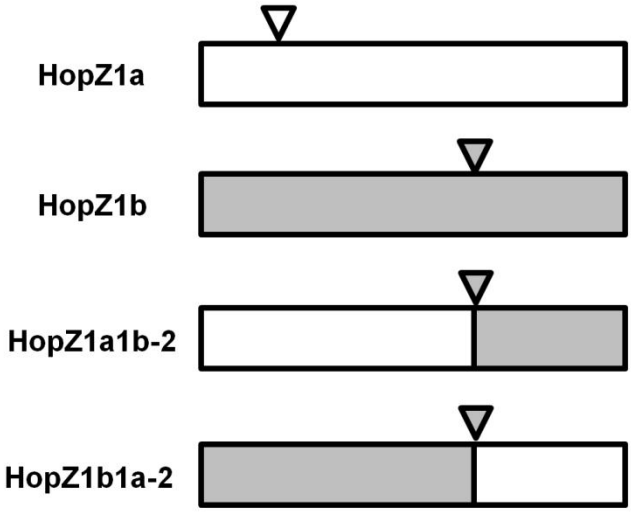
Figure 1.24. Autoacetylation activities of HopZ1a, HopZ1b, and their chimeric proteins.

(A) Constructions of HopZ1 chimeric proteins. All the genes that encode HopZ1 chimeric proteins were constructed in pRSF vector with 8×HIS and SUMO tags in the N-terminus. The open and grey triangles represent the two major indels: 28–31 aa of HopZ1a and 251–253 aa of HopZ1b, respectively. HopZ1a1b-2 has HopZ1a1–244 aa fused to HopZ1b240–368 aa; HopZ1b1a-2 has HopZ1b1–239 aa fused to HopZ1a245–369 aa.

(B) HopZ1b1a-2 gained autoacetylation activity although HopZ1a1b-2 exhibited the same acetylation activity as HopZ1a. HopZ1a, HopZ1b, HopZ1a1b-2 and HopZ1b1a-2 were purified using nickel resins. They were mixed with AtJAZ6 and subjected to autoradiograph.

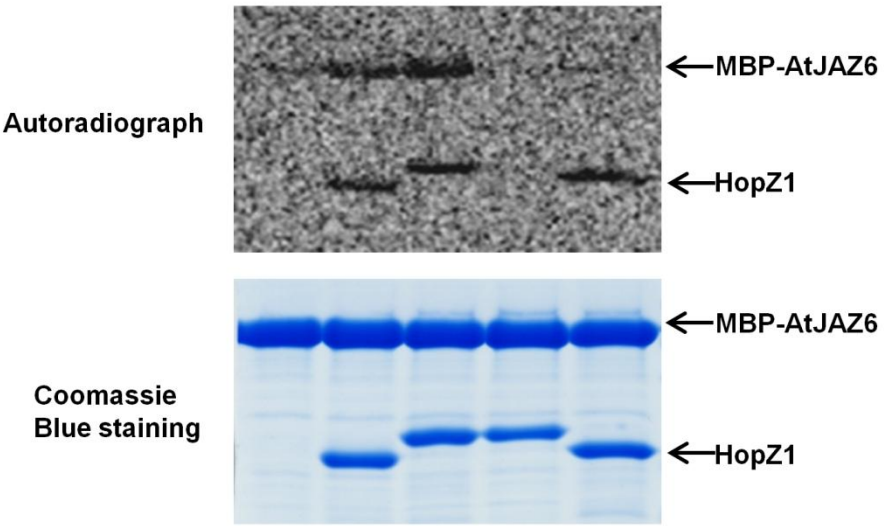
These experiments were repeated twice with similar results.

A



B

AtJAZ6	+	+	+	+	+
HopZ1a		+			
HopZ1a1b-2			+		
HopZ1b				+	
HopZ1b1a-2					+



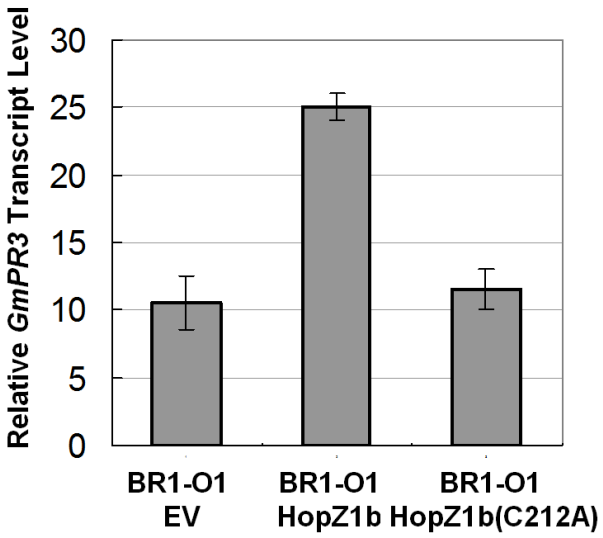
HopZ1b activates JA signaling in soybean

As HopZ1b triggered the degradation of GmJAZ1 when they were co-expressed in *N. benthamiana* (Figure 1.9), I tested whether HopZ1b could also activate JA signaling and induce JA-responsive gene expression during bacterial infection. Real-time PCR was applied to determine the transcript levels of the JA/ET-responsive gene *GmPR3* (Mazarei et al, 2007) and the JA-inducible gene *GmVSPA* (Mason & Mullet, 1990) in soybean nine hours after the plants were inoculated by *P. syringae* pv. *glycinea* strain BR1-O1 (*Pgy*BR1-O1) expressing HopZ1b or HopZ1b(C212A) (OD_{600} = 0.2). *GmPR3* and *GmVSPA* were induced to approximately two and six folds in leaves infected with *Pgy*BR1-O1 expressing HopZ1b, comparing to the transcript levels in leaves inoculated with *Pgy*BR1-O1 expressing HopZ1b(C212A) or carrying the empty vector (Figure 1.25). These data demonstrate that HopZ1b also activates JA signaling in soybean.

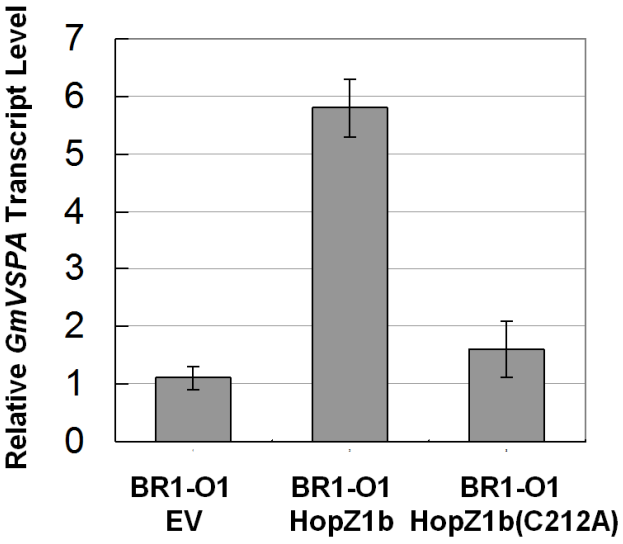
Figure 1.25. HopZ1b induces the expression of the JA-responsive genes *GmPR3* and *GmVSPA* in soybean.

Soybean unifoliates were inoculated with *Pgy*BR1-O1 carrying the empty vector pUCP20tk (EV), pUCP20tk::HopZ1b-HA or pUCP20tk::HopZ1b(C212A)-HA. *GmPR3* and *GmVSPA* transcript levels were analyzed at nine hours post inoculation by real-time RT-PCR using *GmUBI* as the internal standard. Relative expression levels were determined by comparing the normalized *GmPR3* **(A)** or *GmVSPA* **(B)** transcripts between infected and mock-treated (leaves infiltrated with 10 mM $MgSO_4$) samples.

A


Relative GmPR3 Transcript Level
30
25
20
15
10
5
0
BR1-O1 EV
BR1-O1 HopZ1b
BR1-O1 HopZ1b(C212A)

B


Relative GmVSPA Transcript Level
7
6
5
4
3
2
1
0
BR1-O1 EV
BR1-O1 HopZ1b
BR1-O1 HopZ1b(C212A)

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 (Robert-Seilaniantz et al, 2007).

SA-dependent defense plays a major role in plant immunity against the biotrophic and hemibiotrophic pathogens, such as *Hyaloperonospora arabidopsidis* and *P. syringae* (Robert-Seilaniantz et al, 2007; Zheng et al, 2012). The *P. syringae* effector HopI1 directly targets Hsp70 in choloroplasts to suppress SA accumulation and thereby facilitates bacterial infection (Jelenska et al, 2007). In addition, the *Xanthomonas campestris* effector XopD was also shown to repress SA signaling during bacterial infection of tomato (Kim et al, 2013; Kim et al, 2008; O'Donnell et al, 2001). In this thesis, my research demonstrated that the *P. syringae* effector HopZ1a represses SA accumulation, probably as an indirect effect of the activation of JA signaling, in *Arabidopsis*.

In this study, I showed that HopZ1 directly targets JAZs, the key negative regulators of the JA signaling (Chini et al, 2007; Thines et al, 2007). Because the 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. 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 (Katsir et al, 2008; Sheard et al, 2010). However, it has been rather puzzling that only a few *P.*

syringae strains produce coronatine (Hwang et al, 2005). A previous report showed that a T3SE, AvrB, was also able to promote JA signaling, apparently through an indirect mechanism via the activation of MPK4 (Cui et al, 2010; He et al, 2004). My experiments show that the effector HopZ1 directly interacts with JAZs, and at least some of the 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 AtJAZ3 (Mukhtar et al, 2011). These pieces of evidence suggest that the JA 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 *Pto*DC3118, but not that of *Pto*DC3000, in *Arabidopsis*. My 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 (Zeng et al, 2011), whereas the type III secretion genes are generally believed to be expressed after the bacteria have entered the apoplast (Rico & Preston, 2008). 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 *Pto*DC3118.

As transcription regulators, JAZs are believed to function in the nucleus. However, HopZ1a was previously shown to mainly locate on the plasma membrane and this localization was mediated by a potential myristoylation site in the N-terminus (Lewis et al, 2008). My 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 *Pto*DC3118 infection. These data demonstrated that the membrane localization of HopZ1a is only important for host recognition (Lewis et al, 2008; Zhou et al, 2009), but not required for the virulence activity. This is consistent with the primary localization of JAZs in the nucleus as transcription repressors.

YopJ-like T3SEs produced by plant pathogens appear to have various enzymatic activities. AvrXv4 of *Xanthomonas campestris* was reported to be a SUMO protease (Roden et al, 2004). AvrBsT, also from *Xanthomonas*, exhibited a weak cysteine protease activity in vitro (Szczesny et al, 2010). PopP2 in *R. solanacearum* has autoacetylation and trans-acetylation activities in vitro, but it does not seem to acetylate its host target proteins (Tasset et al, 2010). Recently, HopZ1a was demonstrated to acetylate tubulin (Lee et al, 2012). My experiments showed that GmJAZ1 and AtJAZ6 are also substrates of HopZ1a. Importantly, I 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 (Chini et al, 2007; Katsir et al, 2008; Thines et al, 2007). 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 (Jeong et al, 2002). Further investigations are needed to determine how HopZ1a-mediated acetylation of JAZs could facilitate COI1-dependent degradation of JAZ repressors.

HopZ1a and HopZ1b shares high similarity in protein sequences. However, unlike HopZ1a, HopZ1b does not have acetyltransferase activity in the in vitro experiment condition. The chimeric protein HopZ1b1a2 constructed by domain shuffling gained autoacetylation activity but was still unable to acetylate AtJAZ6. These data indicate that the sequence difference of HopZ1a and HopZ1b may directly or indirectly impact their enzymatic activities. It is possible that certain amino acid residues in the C-terminal domain (HopZ1a245–369 aa) play important roles in the autoacetylation of HopZ1a. As a result, HopZ1a245–369 aa conferred HopZ1b the autoacetylation ability. Target mutagenesis analysis might help to find key amino acids that are responsible for HopZ1a autoacetylation. Nonetheless, there might be additional potential acetylation sites within the N-terminal half of HopZ1a (1-244 aa), because HopZ1a1b-2 acts just like HopZ1a. The result that HopZ1b1a-2 was unable to acetylate AtJAZ6 might be explained by differences between HopZ1a and HopZ1b in

their interactions with JAZs, or sequence difference in HopZ1 that impact the transacetylation activity. Crystal structures of HopZ1a and HopZ1b will be important to analyze the differences of their enzymatic activity in the future.

As HopZ1b was reported to cause weak cell death in *Arabidopsis*, most of my experiments with HopZ1b were conducted in soybean. My results clearly showed that HopZ1b interacts with GmJAZ1 in the in vitro pull-down assay and in vivo BiFC assay. Furthermore, HopZ1b activates JA signaling during bacterial infection in soybean. However, more experiments are needed in the future to study the detailed mechanisms underlying HopZ1b-JAZ interaction. For example transgenic soybean plants are needed to examine whether HopZ1b degrades GmJAZ1 during bacterial infection. As HopZ1b showed a different enzymatic activity as HopZ1a, further investigations on the enzymatic activity of HopZ1b are needed to understand how HopZ1b promotes JA degradation.

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Chapter 2

Molecular Mechanisms of HopZ1a-Mediated Acetylation of JAZs

ABSTRACT

HopZ1a is a type III secreted effector (T3SE) of *Pseudomonas syringae* that activates the Jasmonate signaling in plants and promotes bacterial infection. My experiments showed that HopZ1a directly interacts with multiple jasmonate ZIM-domain (JAZ) proteins in soybean and *Arabidopsis* and triggers their degradation. Furthermore, HopZ1a modifies JAZs as an acetyltransferase in vitro. However, detailed molecular mechanisms underlying how the acetylation of JAZs may lead to their degradation and benefit bacterial infection remain unclear. To begin addressing these questions, I conducted experiments to survey AtJAZs that can be acetylated by HopZ1a. My data showed that, in addition to AtJAZ6, HopZ1a also acetylates AtJAZ8, AtJAZ9 and AtJAZ10 in vitro. Because AtJAZ8 can be strongly acetylated by HopZ1a, I determined that the TSYDSDSSDTTS peptide in the close proximity of an ERF-associated amphiphilic repression (EAR) motif in the N-terminal region of AtJAZ8, contains the acetylation site(s) of AtJAZ8. The AtJAZ8 mutants AtJAZ8Δ1 with the TSYDSDSSDTTS peptide deleted or AtJAZ8m1 with all the eight serine and threonine residues within this region substituted with alanines exhibited drastically decreased levels of acetylation by HopZ1a. However, two mutants with single substitutions of one of the threonine residues were still able to be acetylated. These data suggest that HopZ1a may acetylate one or multiple serine/threonine residues within this region.

INTRODUCTION

Jasmonates (JA), since their discovery in 1960s, have been gradually recognized as essential plant hormones that benefit plant fitness against the changing environment. They play important roles in multiple biological processes, including root growth, plant development, and plant responses to biotic and abiotic stresses (Browse, 2009; Glazebrook, 2005; Howe & Jander, 2008; Wasternack, 2007). The identification of the Jasmonate ZIM-domain (JAZ) proteins and their interacting transcription factors represents a significant advance in the understanding of how JA signaling modulates a broad range of gene expressions involved in these various plant processes (Chini et al, 2007; Pauwels & Goossens, 2011; Thines et al, 2007; Yan et al, 2007).

JAZs are key negative transcription regulators of JA-responsive genes and important component of the JA receptor complex (Chini et al, 2007; Sheard et al, 2010; Thines et al, 2007; Yan et al, 2007). JAZs belong to the plant-specific TIFY family of transcription regulators that are characterized by the presence of a TIF[F/Y]XG motif within a conserved region (~28 amino acids) designated as the TIFY (ZIM) domain (Bai et al, 2011; Vanholme et al, 2007). Unlike other TIFY transcriptional regulators, JAZs contain a multifunctional Jas domain in the C-termini but lack a known DNA binding domain; therefore interactions with other transcription factors are required for JAZs to function as transcription regulators (Vanholme et al, 2007). So far, several R2R3 MYB transcription factors (e.g. MYB75) and bHLH transcription factors (e.g. MYC2, MYC3 and MYC4) have been shown to associate

with JAZ and activate JA-responsive gene expression (Cheng et al, 2011; Chini et al, 2007; Fernandez-Calvo et al, 2011; Niu et al, 2011; Qi et al, 2011; Song et al, 2011).

The Jas domain is also critical for the proteasomal degradation of JAZs in the presence of the main active form JA-Ile or the bacterial phytotoxin coronatine, which is a structural mimic of JA-Ile (Chini et al, 2007; Thines et al, 2007). JA-Ile or coronatine promote the binding of JAZs with an F-box protein COI1, and trigger the subsequent unbiquitination and degradation of JAZs via the 26S proteasome pathway (Katsir et al, 2008; Yan et al, 2009). Several lines of evidence suggest that the Jas domain is essential for JAZ-COI1 interaction (Melotto et al, 2008).

The *Arabidopsis* genome encodes 12 JAZs (JAZ1 to JAZ12). Recent research revealed interesting differences among these AtJAZs, especially in their binding affinities with COI1. Structural analysis unraveled a bipartite degron within the N-terminal half of the Jas domain. This structure consists of a loop region that interacts with both JA-Ile and COI1 and an α-helix that docks JAZs on COI1 (Sheard et al, 2010). Site-directed mutagenesis revealed two arginine residues in the loop region that are essential for the interaction of AtJAZ1 and AtJAZ9 with COI1 (Melotto et al, 2008).

This N-terminal loop of Jas domain is relatively variable among the 12 AtJAZs; on the contrary, the α-helix (SLX2FX2KRX2R) region and the C-terminal (X5PY) region of Jas domain are highly conserved (Sheard et al, 2010; Shyu et al, 2012). Members belong to the major subgroup of AtJAZs (i.e. AtJAZ1, AtJAZ2, AtJAZ3 and AtJAZ10) contain the canonical LPIAR(R/K) motif in the loop region and exhibit robust interaction with COI1 in the presence of JA-Ile (Chini et al, 2007; Thines et al,

2007). However, the loop regions in AtJAZ5 and AtJAZ6 do not have the conserved N-terminal LP residues; thereby, although direct interaction has been shown between COI1 and AtJAZ6 (Sheard et al, 2010; Shyu et al, 2012; Thines et al, 2007), AtJAZ6 seemed to have reduced binding affinity with COI1, compared to AtJAZ2, AtJAZ3, AtJAZ10 and AtJAZ12, in a pull-down assay (Shyu et al, 2012). AtJAZ8 contains the X3SMK residues, instead of canonical LPIAR(R/K) residues, in the loop region and does not associate with COI in vitro even in the presence of high concentrations of JA-Ile (Shyu et al, 2012). Weak protein-protein interactions between COI1 and AtJAZ8 were only observed in the presence of high concentrations of coronatine, which is 100-1000 folds more effective in recruiting JAZs to COI1 compared to JA-Ile (Shyu et al, 2012). Moreover, *AtJAZ8* over-expression *Arabidopsis* plants are resistant to JA-mediated degradation and insensitive to JA-triggered root growth inhibition (Lorenzo et al, 2004; Shyu et al, 2012). These results support that AtJAZs have different degrees of stability in the presence of JA/coronatine, and hence may be regulated through different mechanisms. This could primarily be due to sequence variability in the loop regions within the Jas domains.

Most JAZs interact with the NOVEL INTERACTOR OF JAZ (NINJA) protein via the central ZIM domain. NINJA also associates with the Groucho/Tup1 transcription co-repressor TOPLESS (TPL), as well as its homologues TPR2 and TPR3 (Pauwels et al, 2010; Pauwels & Goossens, 2011), through an ETHYLENE RESPONSE FACTOR (ERF)–associated amphiphilic repression (EAR) motif. Together with these co-repressors, JAZs suppress JA signaling (Pauwels et al, 2010). On the contrary, AtJAZ8 weakly binds NINJA, and the ZIM domain is not required

for AtJAZ8-mediated repression of JA-induced root growth inhibition (Shyu et al, 2012). Instead, AtJAZ8 contains an LxLxL-type EAR motif that locates to the N-terminus of the protein (Bai et al, 2011; Kagale et al, 2010). This EAR motif contributes to a direct interaction of AtJAZ8 with the co-repressor TPL to confer transcription repression (Shyu et al, 2012). It is therefore evident that different strategies are used by different JAZs to modulate JA signaling.

HopZ1 belongs to the YopJ family of type III-secreted effectors (T3SEs), which are widely distributed in both plant and animal pathogens (Hinnebusch, 2005; Lewis et al, 2011; Ma et al, 2006). Histidine, glutamic acid, and cysteine are three key amino acid residues within the catalytic core of the YopJ family effectors and are required for both the virulence and avirulence functions of these effectors (Lewis et al, 2011; Ma et al, 2006; Zhou et al, 2009). Several YopJ family effectors have been reported to function as acetyltransferase. YopJ of *Yersinia pestis* acetylates critical serine/threonine residues in the activation loop of MKK6, MKK1/2, IKKα/β and TAK1, which are important kinases involved in MAPK or NF-κB signaling pathways (Mittal et al, 2006; Mukherjee et al, 2006; Paquette et al, 2012). The acetylations of these key enzymes block their phosphorylation and the downstream signal propagation. Furthermore, another YopJ family effector VopA acetylates a conserved lysine residue, in addition to serine and threonine residues, in the catalytic loop of MAPK kinases in order to inhibit ATP binding and thereby blocking MAPK signaling (Trosky et al, 2004), (Trosky et al, 2007).

Data from my research and other labs suggest that HopZ1a also possesses an acetyltransferase activity using tubulin (Lee et al, 2012) and JAZs as the substrates in

vitro (Jiang et al, 2013). My experiments also showed that HopZ1a could induce the degradation of JAZs (Jiang et al, 2013). However, the molecular mechanisms underlying how the acetylation of JAZs can lead to their degradation and contribute to bacterial infection remain unclear.

To address this question, I investigated the acetylation of eight AtJAZs by HopZ1a using purified proteins. In vitro experiments showed that, in addition to AtJAZ6, AtJAZ8, AtJAZ9 and AtJAZ10 are also substrates of HopZ1a that could be strongly acetylated. I also confirmed that HopZ1a physically interacted with AtJAZ8 using in vitro pull-down and in vivo BiFC assays. Furthermore, I collaborated with Dr. Gitta Coaker at UC Davis and mapped the acetylation site(s) in AtJAZ8. Mass spectrometry analyses revealed the peptide TSYDSDSSDTTS (AtJAZ8 16-27 aa) that contains the acetylation sites. AtJAZ8 mutant (AtJAZ8Δ1) with this motif deleted still interacts with HopZ1a, but can no longer be acetylated by HopZ1a. Unfortunately, I could not detect the degradation of AtJAZ8 by HopZ1a using *Agrobacterium*-mediated transient expression in *Nicothiana benthamiana*. I am in the process of generating *Arabidopsis* transgenic plants expressing *AtJAZ8, AtJAZ8Δ1* or *AtJAZ8m1* (an AtJAZ8 mutant with all the serine and threonine residues within the acetylated peptide replaced with alanines) in the *zar1-1* background. These plants will be used to test the activation of JA signaling, in vivo AtJAZ8 degradation, as well as bacterial infection. These experiments will help elucidate the biological consequences of HopZ1a-mediated acetylation of AtJAZ8.

MATERIALS and METHODS

Bacterial strains and plasmids

Agrobacterium tumefaciens and *Escherichia coli* were grown in Luria–Bertani (LB) medium (BD Co., Franklin Lakes, NJ, USA) with appropriate antibiotics at 30°C or 37°C respectively. Plasmids were transformed into *A. tumefaciens* and *E. coli* chemical competent cells as previously described (Morgan et al, 2010). All the bacterial strains and plasmids used in this study were listed in table 2.1.

Table 2.1. Bacterial strains and plasmids used in the study in chapter 2.

Strains or Plasmids	Description	Source/ reference
Escherichia coli DH5α	F- Φ80d*lacZ*ΔM15 Δ(*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1, *hsd*R17(rk-, mk+) *pho*A *sup*E44 λ- *thi*-1 *gyr*A96 *rel*A1	Invitrogen
Escherichia coli BL21(DE3)	F^- *ompT gal dcm lon hsd*S_B(r_B^- m_B^-) λ(DE3 [*lacI lacUV5-T7 gene 1 ind1 sam7 nin5*]	Invitrogen
Agrobacterium tumefaciens GV3101(pMP90)	Rif^R, $Gent^R$	Holsters, 1980
Agrobacterium tumefaciens C58C1 (pCH32)	Rif^R, Tet^R	Mudgett et al., 2000
pEG100	pEarleyGate100, a Gateway binary vector with cauliflower mosaic virus *35S* promoter, Kan^R	Earley et al., 2006
pEG100::*3×FLAG-HopZ1a*	pEG100 carrying *hopZ1a* tagged with 3×FLAG at N-terminus, Kan^R	This study
pEG100::*3×FLAG-HopZ1a(C216A)*	pEG100 carrying *hopZ1a(C216A)*tagged with 3×FLAG at N-terminus, Kan^R	This study
pEG101	pEarleyGate101, a Gateway binary vector for YFP fusion protein expression with cauliflower mosaic virus *35S* promoter, Kan^R	Earley et al., 2006
pEG101::*AtJAZ8*	*AtJAZ8* is in-frame fused to YFP and HA, Kan^R	This study
pEG101::*AtJAZ8Δ1*	*AtJAZ8Δ1* (with 14 aa deletion from Pro^{15} to Val^{28}) is in-frame fused to YFP and HA, Kan^R	This study
pSPYNE	A binary vector with cauliflower mosaic virus *35S* promoter and the N-terminal (1-155 aa) domain of YFP (nYFP), Kan^R	Walter et al., 2004
pSPYCE	A binary vector with cauliflower mosaic virus 35s promoter and the C-terminal (156-239 aa) domain of YFP (cYFP), Kan^R	Walter et al., 2004
pSPYNE::*hopZ1a(C216A)*	pSPYNE carrying *hopZ1a(C216A)* in-frame fused with nYFP, Kan^R	Zhou et al., 2011
pSPYCE::*AtJAZ8*	pSPYCE carrying *AtJAZ8* in-frame fused with cYFP, Kan^R	This study
pGEX4T-2	*E. coli* expression vector with an N-terminal GST tag, Amp^R	Amersham
pGEX4T-2::*hopZ1a*	pGEX4T-2 carrying *hopZ1a*, Amp^R	Zhou et al., 2011
pET-mal	*malE* gene from pMAL-c2 is cloned into *Nde*I-*Xho*I site of pET28a, Kan^R	Sweeney et al., 2005
pET-mal::*AtJAZ*	pET-mal carrying *AtJAZ* genes, Kan^R	Sheng Yang He
pET-mal::*AtJAZ8Δ1*	pET-mal carrying *AtJAZ8Δ1* (with 14 aa deletion from Pro^{15} to Val^{28}), Kan^R	This study
pET-mal::*AtJAZ8m1*	pET-mal carrying *AtJAZ8m1* gene encoding AtJAZ8 mutant with thr^{16}, ser^{17}, ser^{20}, ser^{22}, ser^{23}, thr^{25}, thr^{26}, ser^{27} replaced with alanines, Kan^R	This study

pET-mal::*AtJAZ8m2*	pET-mal carrying *AtJAZ8m2* gene encoding AtJAZ8 mutant with thr^{25} replaced with an alanine, Kan^R	This study
pET-mal::*AtJAZ8m3*	pET-mal carrying *AtJAZ8m3* gene encoding AtJAZ8 mutant with thr^{16} replaced with an alanine, Kan^R	This study
pENTR/D-TOPO	An entry vector for the Gateway system, Kan^R	Invitrogen
pENTR/D-TOPO::*3XFLAG-hopZ1a*	pENTR/D-TOPO carrying the *hopZ1a* gene with a 3XFLAG tag at the N-terminus, Kan^R	This study
pENTR/D-TOPO::*3XFLAG-hopZ1a(C216A)*	pENTR/D-TOPO carrying the *hopZ1a(C216A)* gene with a 3XFLAG tag at the N-terminus, Kan^R	This study
pSITE2CA	binary vector, carrying the *GFP* gene, Kan^R	Chakrabarty et al., 2007
pSITE2CA::*AtJAZ8*	pSITE2CA carrying *AtJAZ8* to make a N-terminal fusion of AtJAZ8 with GFP,	Sheng Yang He

In vitro acetylation assays

HIS-SUMO-HopZ1a, HIS-SUMO-HopZ1a(C216A), MBP-AtJAZ2-HIS, MBP-AtJAZ5-HIS, MBP-AtJAZ7-HIS, MBP-AtJAZ8-HIS, MBP-AtJAZ8Δ1-HIS (a deletion mutant of AtJAZ8 from Pro^{15} to Val^{28}), MBP-AtJAZ8m1-HIS (an AtJAZ8 mutant with Thr^{16}, Ser^{17}, Ser^{20}, Ser^{22}, Ser^{23}, Thr^{25}, Thr^{26}, Ser^{27} replaced with alanines), MBP-AtJAZ8m2-HIS (an AtJAZ8 mutant with Thr^{25} replaced with an alanine), MBP-AtJAZ8m3-HIS (an AtJAZ8 mutant with Thr^{16} with an analine), MBP-AtJAZ9-HIS, MBP-AtJAZ10-HIS, MBP-AtJAZ11-HIS and MBP-AtJAZ12-HIS were over-expressed in the *E. coli* strain BL21(DE3) and then purified using nickel resins. After one-hour incubation, the nickel resins were washed five times using washing buffer (50 mM Tris-HCl pH=8.0, 1 M NaCl, 20 mM immidazole), and then the proteins were eluted using an elution buffer (50 mM Tris-HCl pH=8.0, 1 M NaCl, 200 mM immidazole). HIS-SUMO-HopZ1a and HIS-SUMO-HopZ1a(C216A) proteins were cleaved by ULP1 protease to remove the HIS tag. The protein mixture after digestion was incubated with nickel resins again, and the tag-free HopZ1a or HopZ1a(C216A) proteins were collected from the flow through. All the purified proteins were dialyzed into a low salt solution (20 mM Tris-HCl pH=7.5, 200 mM NaCl, 5 mM DTT/β-mercaptethanol) at 4°C for overnight and then stored with glycerol at a final concentration of 10% at -80°C.

In a standard acetylation assay, 2 μg of purified HopZ1a or HopZ1a(C216A) was incubated with 10 μg MBP-AtJAZ-HIS at 30°C for one hour in a 25 μL reaction system containing 50 mM HEPES (pH 8.0), 10% glycerol, 1 mM DTT, 1 mM PMSF, 10 mM sodium butyrate, 1 μL [14C]-acetyl-CoA (55 μci/μmol, Amersham) and 100

nM inositol hexakisphosphate (IP6, Sigma). The reaction mixtures were subjected to SDS-PAGE and the acetylated proteins were detected by autoradiography after exposure at -80°C for five days (Mittal et al, 2006; Mittal et al, 2010; Mukherjee et al, 2006). After autoradiograph, the protein gels were removed from the filter paper and stained with Coomassie blue as a loading control.

Mass spectrometry

To prepare the samples for mass spectrometry analysis, 2 μg purified HopZ1a or HopZ1a(C216A) was incubated with 10 μg MBP-AtJAZ8-HIS at 30°C for one hour in 25 μL reaction systems, which contained the same chemicals as in the above-described acetylation assays except that 1 μL of 2 mM acetyl-CoA sodium salt (sigma) was used to replace the 1 μL of [14C]-acetyl-CoA. The reaction mixes were then subjected to the SDS-PAGE and stained by Coomassie blue. Protein bands containing AtJAZ8 were excised from the gel and sent to UC Davis for Mass Spectrometry analysis.

In vitro GST pull-down assays

To prepare the plasmids required for GST pull-down assay, the full-length *hopZ1a* gene was inserted into the vector pGEX4T-2 (GE Healthcare Life Science). The pET-MAL::*AtJAZ8* construct was obtained from Dr. Sheng Yang He at Michigan State Univrsity. pET-MAL vector contains DNA sequences that encode the maltose binding protein (MBP) in the N-terminus and 8×His in the C-terminus of the inserted genes (Sweeney et al, 2005). pET-MAL::*AtJAZ8Δ1* was constructed by ligating a linear

PCR product using pET-14b::*AtJAZ8* as the template and a pair of primers designed to delete twelve amino acids from thr^{16} to ser^{27}. The recombinant plasmids were then introduced into *E. coli* strain BL21(DE3).

Large-scale protein induction protocol as described in Chapter 1was used to induce the expression of GST- HopZ1a, MBP-AtJAZ8-HIS and MBP-AtJAZ8Δ1-HIS, which were then purified using nickel resins. In vitro pull-down assays were carried out using ProFound^{TM} pull-down GST protein:protein interaction kit (Pierce) according to the manufacturer's instruction. Briefly, after sonication (performed 4-5 cycles using the Cell Disruptor 350 (Branson Sonic Power Co.) at power setting 5 and 50% duty cycle), soluable cell lysates containing GST-HopZ1a were incubated with 50 µL of glutathione agarose beads (Invitrogen) for one hour at 4°C. The beads were washed five times in a washing buffer (20 mM Tris-HCl (pH=7.5), 150 mM KCl, 0.1 mM EDTA and 0.05% Triton X-100) for five times and then incubated with equal amount of purified AtJAZ8 or AtJAZ8Δ1 at 4°C for overnight. The beads were washed five times again, and the presences of the AtJAZ8 or AtJAZ8Δ1 proteins on the beads were detected by western blots using anti-HIS antibodies conjugated with horseradish peroxidase (HRP) (Santa Cruz Biotechnology Inc.).

Fluorescence microscopy

To test the interaction of AtJAZ8 and HopZ1a(C216A) using bimolecular fluorescence complementation (BiFC) assay, full-length cDNA of AtJAZ8 and hopZ1a(C216A) were cloned into the vectors pSPYCE and pSPYNE (Walter et al, 2004), respectively. The recombinant plasmids were introduced into *A. tumefaciens*

strain C58C1(pCH32) (Mudgett et al, 2000), which were used to infiltrate 3-4 week old *N. benthamiana* plants as described in Chapter 1. *Agrobacterium* strains expressing P19, a viral suppressor of RNA silencing (Chu et al, 2000), was used to co-infiltrate *N. benthamiana* leaves with the *Agrobaterium* strains expressing HopZ1a(C216A)-nYFP and AtJAZ8-cYFP. The final concentrations demonstrated by OD_{600} values for each bacterium suspensions in the infiltration solution were 0.33 (for P19), 0.5 (for HopZ1a) and 0.5 (for AtJAZ8).

pSITE2CA::*AtJAZ8* construct was provided by Dr. Sheng Yang He at Michigan State University to examine the subcellular localization of AtJAZ8. pSITE2CA is a binary vector which carries DNA sequences that encode the GFP protein that can be fused in frame with the insertion sequence (Chakrabarty et al, 2007). pSITE2CA::*AtJAZ8* was transformed into *A. tumefaciens* strain GV3101 (Holsters et al, 1980), which was next used to infiltrate *N. benthamiana* plants using a needle-less syringe at a final OD_{600}=0.5.

The Inoculated plants were kept at room temperature under continuous low light for 48 hours. Functional fluorophore were detected in the infiltrated leaves using a Leica SP2 Laser Scanning Confocal Microscope (Leica Microsystems). DAPI was used to stain the nucleus in plant cells (James & Jope, 1978; Kapuscinski & Skoczylas, 1977).

AtJAZ8 degradation assay

To determine whether AtJAZ8 can be degraded by HopZ1a, *AtJAZ8* and its mutant genes with two nucleotides added at the C-terminus were inserted into entry vector pENTR1a using the *Kpn*I and *Xho*I digestion sites and then were constructed into binary vector pEG101 via Gateway recombinational cloning reactions (Earley et al, 2006). *3×FLAG-HopZ1a* and *3×FLAG-HopZ1a(C216A)* were inserted into pEG100 vector as described in Chapter 1. AtJAZ8-YFP-HA, or its mutants AtJAZ8Δ1-YFP-HA and AtJAZ8m1-YFP-HA were co-expressed with 3×FLAG-HopZ1a or 3×FLAG-HopZ1a(C216A) in *N. benthamiana* using *Agrobacterium*-mediated transient expression as previously described (Ma et al, 2006; Szurek et al, 2001). Leaf tissues were collected at 20 hpi, and then grounded in 2×Laemmli buffer (0.125 M Tris-HCl (PH 6.8), 10% (v/v) β-mercaptoethanol, 4% (w/v) SDS, 20% (v/v) glycerol and 0.04% (w/v) bromophenol blue). Protein abundances of HopZ1a and AtJAZ8 were analyzed by western blots. The protein gels were stained with Coomassie blue as a loading control.

RESULTS

AtJAZ8 and AtJAZ10 are potent acetylation substrates of HopZ1a

Using in vitro pull-down assay, I found that HopZ1a interacts with multiple AtJAZs, and AtJAZ6 was strongly acetylated by HopZ1a (Jiang et al, 2013). To test whether other HopZ1a-interacting AtJAZs could also be the enzymatic substrates of HopZ1a, eight AtJAZ proteins were examined by in vitro acetylation assay using C14-labeled acetyl-CoA.

AtJAZ2, *AtJAZ5*, *AtJAZ7*, *AtJAZ8*, *AtJAZ9*, *AtJAZ10*, *AtJAZ11 and AtJAZ12* cDNAs were cloned in the pET-MAL vector, which allows constructions of JAZ proteins fused with maltose binding protein (MBP) in the N-terminus and 8×His in the C-terminus. The MBP-AtJAZ-HIS proteins were expressed in *E. coli* strain BL21 and purified using nickel resins. 10 µg of MBP-AtJAZ-HIS were incubated with 2 µg of tag-free HopZ1a or HopZ1a(C216A) in the presence of the cofactor inositol hexakisphosphate (IP6). The acetylation of HopZ1a and AtJAZs were then detected by autoradiography.

My results showed that AtJAZ8 and AtJAZ10 were strongly acetylated by HopZ1a (Figure 2.1 A and 2.1 B), although a background level of acetylation was observed in the presence of the catalytic mutant HopZ1a(C216A). AtJAZ9 was also clearly acetylated by HopZ1a, but its acetylation was weaker than that of either AtJAZ8 or AtJAZ10 (Figure 2.1B). On the contrary, HopZ1a were unable to acetylate AtJAZ2, AtJAZ5, AtJAZ7, AtJAZ11 and AtJAZ12 under this in vitro condition (Figure 2.1).

Previously, my in vitro pull-down assay showed that HopZ1a interacts with AtJAZ2, AtJAZ5, AtJAZ6, AtJAZ8 and AtJAZ12, but not with AtJAZ9 and AtJAZ11 (Jiang et al, 2013), whereas my in vitro acetylation assay indicated that HopZ1a acetylated AtJAZ6, AtJAZ8 and AtJAZ10, but not AtJAZ2, AtJAZ5, AtJAZ7, AtJAZ11 and AtJAZ12. Taken together, these experiments showed that AtJAZ6 and AtJAZ8 could both interact with and be acetylated by HopZ1a, AtJAZ11 neither interacts with nor can be acetylated by HopZ1a. However, although HopZ1a bound with AtJAZ2, AtJAZ5 and AtJAZ12, it was unable to acetylate these proteins. The

situation for AtJAZ9 is the opposite. AtJAZ9 did not associate with HopZ1a in the pull-down assay, but was acetylated by HopZ1a in vitro. It is possible that the interaction of AtJAZ9 with HopZ1a is not as strong compared to other AtJAZs, and the weak interaction might not be detectable under the specific experimental protocol that I used. The interactions between HopZ1a and AtJAZ7 or AtJAZ10 have not been examined. In summary, results from in vitro pull-down assays and in vitro acetylation assays suggest differential interaction affinities and acetylating levels of AtJAZs by HopZ1a.

Figure 2.1. HopZ1a acetylates AtJAZ8, AtJAZ9 and AtJAZ10 in vitro.

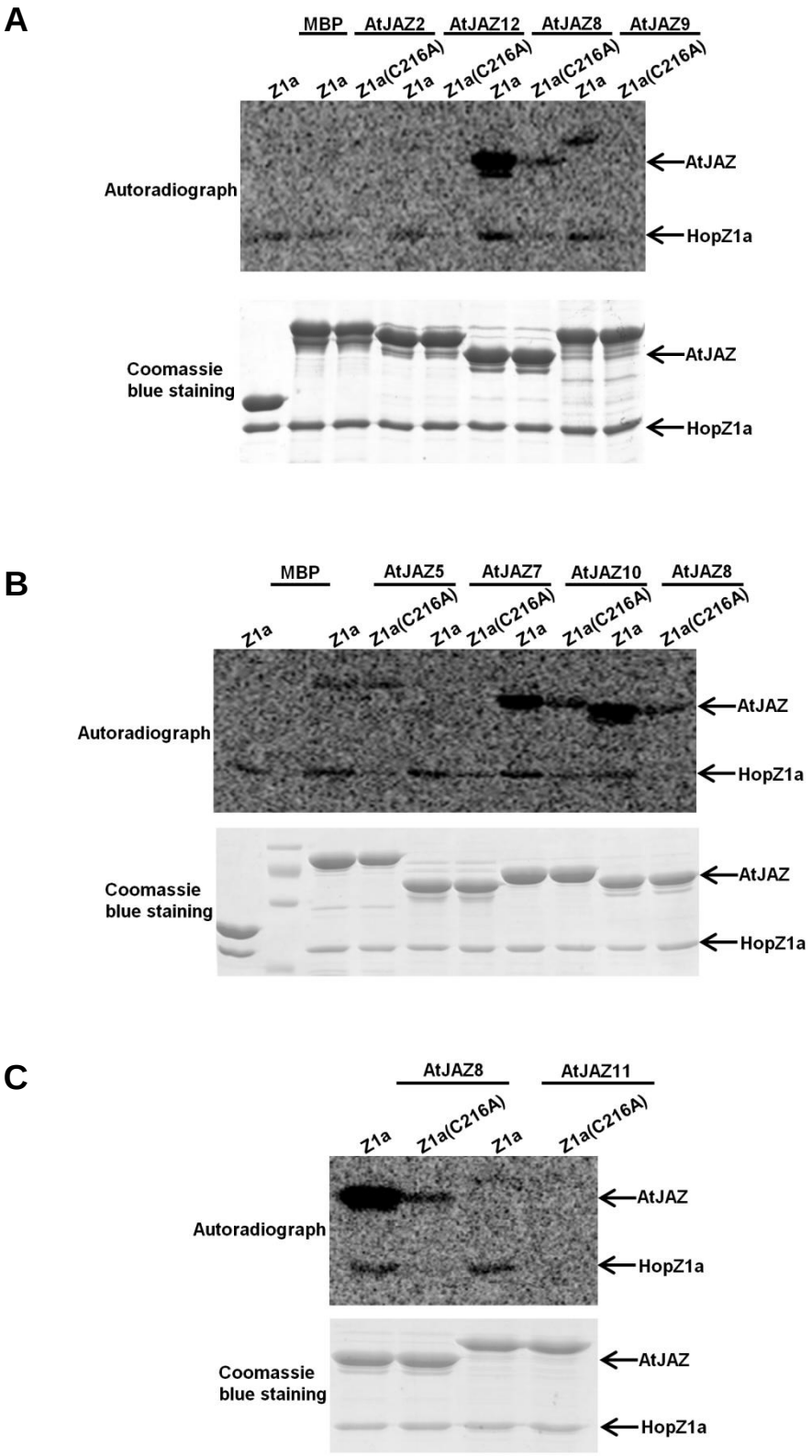
(A) HopZ1a strongly acetylates AtJAZ8, and also acetylates AtJAZ9, but not AtJAZ2 or AtJAZ12 in vitro.

(B) HopZ1a aceylates AtJAZ10, but not AtJAZ5 or AtJAZ7.

(C) HopZ1a cannot acetylate AtJAZ11.

Acetylation of AtJAZs in the presence of HopZ1a or HopZ1a(C216A) was determined using 10 µg of purified AtJAZ proteins and 2 µg of purified HopZ1a. MBP was used as a negative control.

These experiments were repeated twice with similar results.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MBP
AtJAZ2
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AtJAZ8
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HopZ1a physically interacts with AtJAZ8

My previous experiments showed that AtJAZ8 interacts with HopZ1a in vitro (Jiang et al, 2013). However, in those experiments I used the whole cell lysate, rather than purified protein. Therefore, I further confirmed the direct interaction between HopZ1a and AtJAZ8 by in vitro pull-down assay using purified AtJAZ8 proteins.

GST, GST-HopZ1a and MBP-AtJAZ8-HIS were over-expressed in *E. coli*. GST and GST-HopZ1a were purified using glutathione resins, which were then incubated with MBP-AtJAZ8-HIS proteins that were purified using nickel resins. The GST-HopZ1a-bound resins, but not the GST-bound resins, showed the enrichment of AtJAZ8, suggesting that AtJAZ8 specifically and directly associate with HopZ1a (Figure 2.2A).

I then examined the subcellular localization of AtJAZ8 to determine whether it co-localizes with HopZ1a in plant cells. GFP-AtJAZ8 was expressed in *N. benthamiana* using *Agrobacterium*-mediated transient expression. Green fluorescence was detected in the nucleus of the pavement cells of the infiltrated leaves at 48 hours post inoculation (hpi) (Figure 2.2B). These results suggest that AtJAZ8 could co-localize with HopZ1a, which could also enter the nucleus in plant cells. Bimolecular fluorescence complementation (BiFC) assay further confirmed the interaction between AtJAZ8 and HopZ1a in planta. The catalytic mutant HopZ1a(C216A) was used in this experiment, because the expression of the functional HopZ1a triggers cell death in *N. benthamiana* (Ma et al, 2006; Zhou et al, 2009). HopZ1a(C216A) and AtJAZ8 were fused to the C-termini of nonfluorescent N-terminal domain of YFP (1-155 aa, nYFP) and the C-terminal domain of YFP (156-239 aa, cYFP), respectively.

When the recombinant proteins were co-expressed in *N. benthamiana*, yellow fluorescence was mainly detected from the nucleus (Figure 2.2C). In summary, these results suggest that HopZ1a interacts with AtJAZ8 both in vitro and in planta.

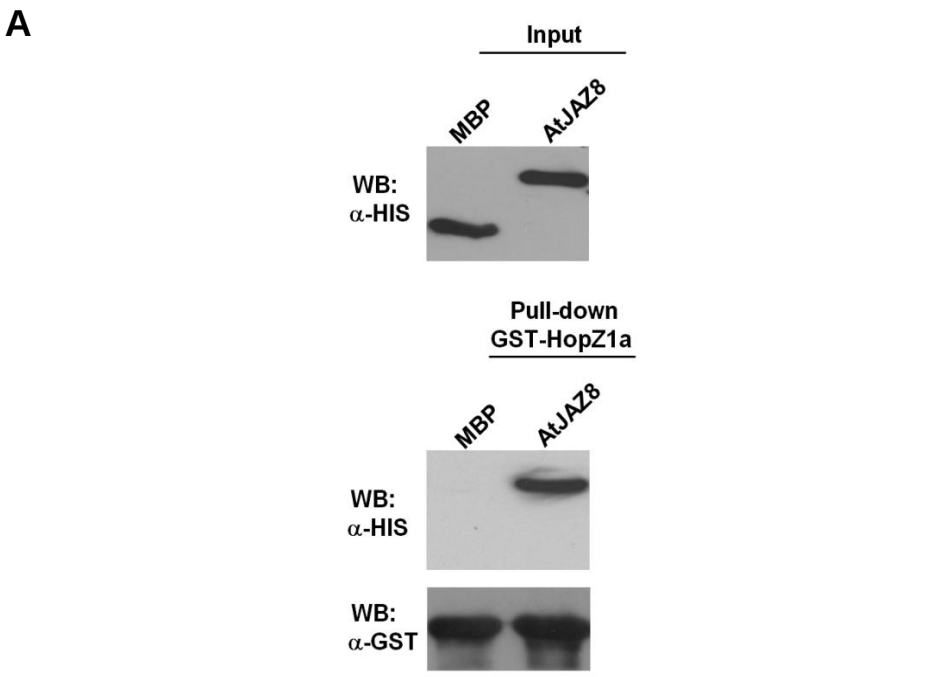
Figure 2.2. HopZ1a interacts with AtJAZ8.

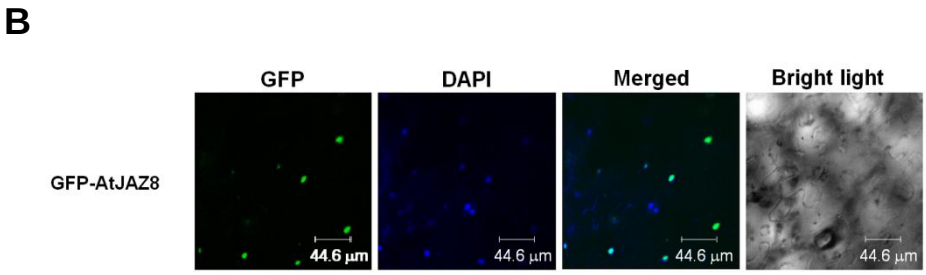
(A) HopZ1a interacts with AtJAZ8 in vitro. GST-HopZ1a, MBP-AtJAZ8-HIS and MBP-AtJAZ8Δ1-HIS were expressed in *E. coli* strain BL21. Co-precipitation of AtJAZ8 with HopZ1a was determined by western blots before (Input) and after affinity purification (Pull-down) using anti-HIS antibody. The protein abundances of MBP-AtJAZ8-HIS on the affinity resins after washes were detected by western blots using anti-HIS antibody.

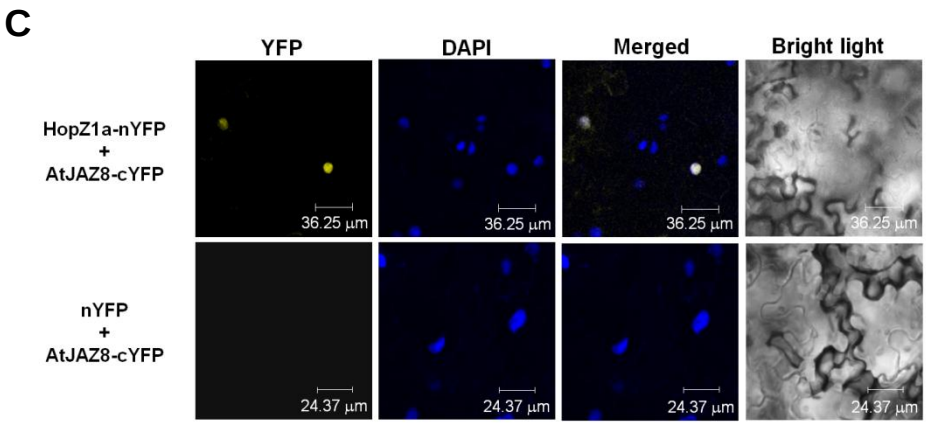
(B) GFP-AtJAZ8 was transiently expressed in *N. benthamiana* leaves to determine its subcellular localization in planta cells. Fluorescence was detected from the infiltrated tissues 48 hours post inoculation using confocal microscopy. DAPI was used to stain the nuclei.

(C) Bimolecular fluorescence complementation analysis of HopZ1a-AtJAZ8 interaction in planta. HopZ1a(C216A)-nYFP and AtJAZ8-cYFP were co-expressed in *N. benthamiana* using *Agrobacterium*-mediated transient expression. Leaves co-infiltrated with *Agrobacterium* carrying AtJAZ8-cYFP + nYFP were used as a negative control.

These experiments were repeated three times with similar results.


A
Input
MBP
AtJAZ8
WB:
α-HIS
Pull-down
GST-HopZ1a
MBP
AtJAZ8
WB:
α-HIS
WB:
α-GST


B
GFP
DAPI
Merged
Bright light
GFP-AtJAZ8
44.6 µm
44.6 µm
44.6 µm
44.6 µm


C
YFP
DAPI
Merged
Bright light
HopZ1a-nYFP
+
AtJAZ8-cYFP
36.25 µm
36.25 µm
36.25 µm
36.25 µm
nYFP
+
AtJAZ8-cYFP
24.37 µm
24.37 µm
24.37 µm
24.37 µm

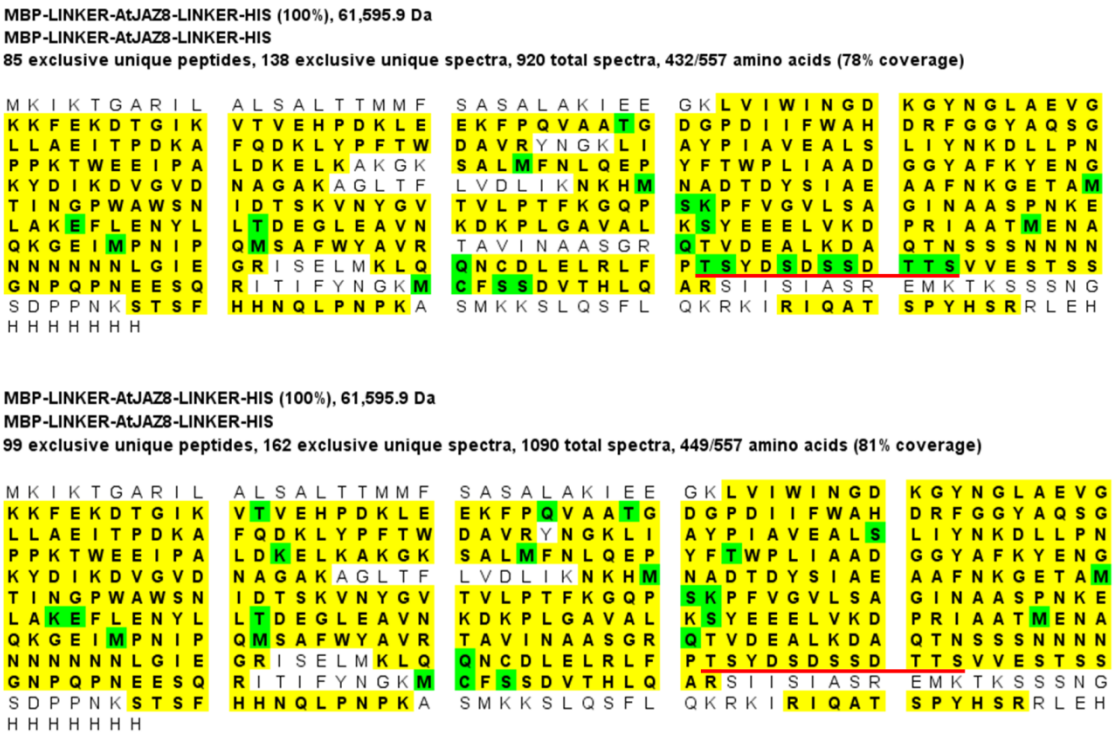
Identification of the acetylation sites of AtJAZ8

Since AtJAZ8 is a potent substrate of HopZ1a, I next investigated the residues in AtJAZ8 that are acetylated by HopZ1a using mass spectrometry analysis. 10 µg AtJAZ8 was mixed with either 2µg HopZ1a or HopZ1a(C216A) in the presence of IP6 and acetyl-CoA sodium salt. Proteins in the reaction mixes were separated on SDS-PAGE and gel slices containing AtJAZ8 were analyzed using Mass Spectrometry by Dr. Gitta Coaker's laboratory at UC Davis.

Figure 2.3 showed all the candidate acetylated residues in AtJAZ8. False positive sites or residues acetylated by other acetyltransferases were excluded from further investigation by comparing the candidate acetylated residues identified from the reaction containing HopZ1a with those identified from the reaction containing HopZ1a(C216A). We paid specific attentions to serine, threonine and lysine residues because they have been reported as the acetylation sites in various targets of other YopJ effectors (Mukherjee et al, 2006; Paquette et al, 2012; Trosky et al, 2007). Based on the mass spectrometry data, I focused on the peptide TSYDSDSSDTTS (from thr^{16} to ser^{27}), which contains a series of serine and threonine residues of AtJAZ8 that seem to be only acetylated by HopZ1a but not HopZ1a(C216A) (Figure 2.3). Interestingly, this sequence is immediately following the LELRL-type EAR motif, which is responsible for the transcription repression function of AtJAZ8 (Shyu et al, 2012). Therefore, the aceyltation of this motif may modulate AtJAZ8 function in suppressing JA signaling.

Figure 2.3. Mass spectrometry analyses of acetylated residues in AtJAZ8 in the presence of HopZ1a or HopZ1a(C216A).

The detectable amino acid residues were highlighted in yellow color. 78% or 81% amino acids of AtJAZ8 were detected in the presence of HopZ1a or HopZ1a(C216A), respectively. The potential acetylated residues of MBP-AtJAZ8-HIS by HopZ1a or HopZ1a(C216A) were highlighted in green color. The underlined TSYDSDSSDTTS peptide were only acetylated by HopZ1a, but not HopZ1a(C216A).



TSYDSDSSDTTS contains the potential acetylation site(s) of AtJAZ8 by HopZ1a

If the TSYDSDSSDTTS peptide contains the potential acetylation site(s), deletion of this region should result in the loss of HopZ1a-mediated AtJAZ8 acetylation. To test this, I constructed *AtJAZ8Δ1* which encodes an AtJAZ8 mutant with Pro^{15} to Val^{28} residues deleted in the pET-MAL vector (Figure 2.4). MBP-AtJAZ8Δ1-HIS was then purified using nickel resins and subjected to in vitro acetylation assay (Figure 2.5). Consistent with the hypothesis that this peptide may contain the acetylation site(s), AtJAZ8Δ1 showed a largely decreased acetylation level, which was similar in the reactions with HopZ1a or HopZ1a(C216A) (Figure 2.5 A), and much lower than the acetylation level of AtJAZ8 by HopZ1a. Importantly, AtJAZ8Δ1 can still interact with HopZ1a, similar to wild type AtJAZ8, in the vitro pull-down assay (Figure 2.6). These results indicate the deletion of this peptide does not interfere with the interaction between AtJAZ8 and HopZ1a, but it is required for its acetylation by HopZ1a.

To further confirm that one or more of the Ser/Thr residues within the TSYDSDSSDTTS peptide are the acetylation sites, I made another AtJAZ8 mutant, AtJAZ8m1, which has all the eight serine and threonine residues replaced with alanines (Figure 2.4). MBP-AtJAZ8m1 was purified using nickel resin as previously described and subjected to in vitro acetylation assay. Similar to AtJAZ8Δ1, the acetylation level of AtJAZ8m1was dramatically decreased, when compared to that of AtJAZ8 (Figure 2.5 A). These results indicate that this TSYDSDSSDTTS region is indeed required for AtJAZ8 acetylation by HopZ1a, mediated by single or multiple serine/threonine residues.

I next performed experiments to determine which serine or threonine residue(s) of AtJAZ8 is the acetylation site(s). According to the mass spectrometry analyses, Thr^{25} and at least one more Ser/Thr within this region are potentially acetylated. Therefore, I constructed AtJAZ8 mutants AtJAZ8m2 to AtJAZ8m9 (Figure 2.4.), each of which has one of eight Ser/Thr residues replaced with an alanine. These mutant proteins were purified and subjected to in vitro acetylation assays. So far, I have examined the mutants AtJAZ8m2 (AtJAZ8(T25A)) and AtJAZ8m3 (AtJAZ8(T16A)), but none of them showed decreased acetylation by HopZ1a (Figure 2.5B and 2.5C). These data suggest that AtJAZ8 is likely acetylated by HopZ1a on more than one residue.

Figure 2.4. Schematic overview of all the AtJAZ8 mutants used in this study.

The substituted amino acid(s) in each AtJAZ8 mutants were highlighted in red.

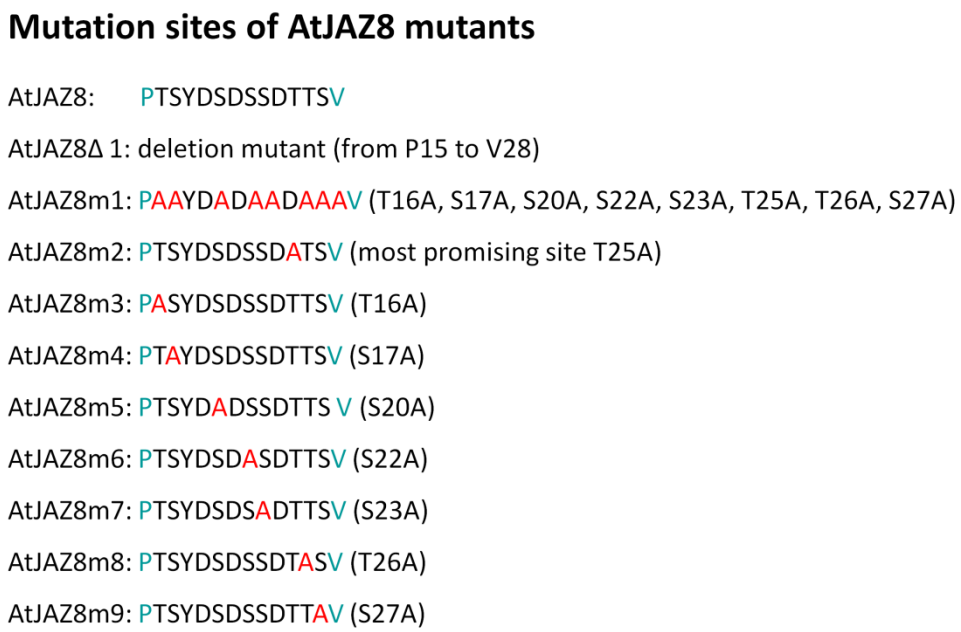
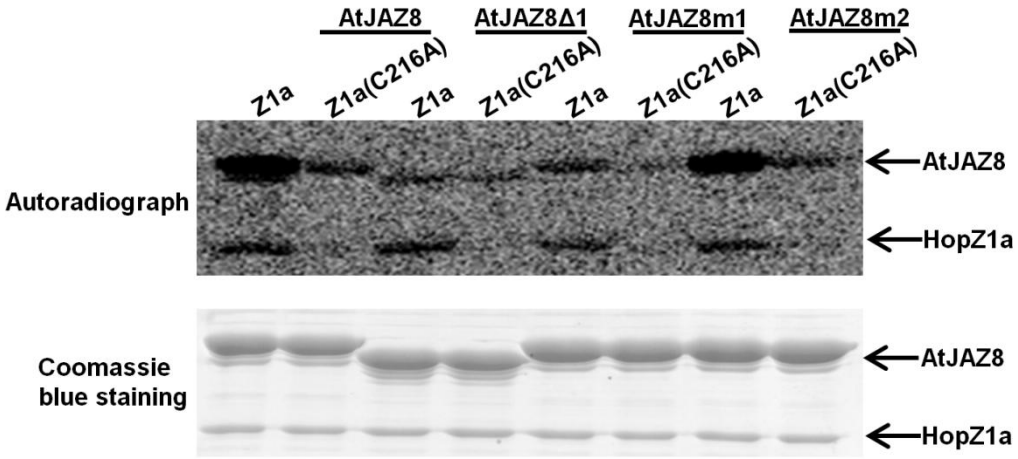


Figure 2.5. AtJAZ8Δ1 and AtJAZ8m1, but not AtJAZ8m2 or AtJAZ8m3,showed decreased acetylation by HopZ1a.

(A) Analyses on the acetylation levels of AtJAZ8Δ1, AtJAZ8m1 and AtJAZ8m2 by HopZ1a. AtJAZ8Δ1showed a largely decreased acetylation level. The aceyltation of AtJAZ8m1 by HopZ1a was strongly reduced when compared with AtJAZ8. Similar to wild type AtJAZ8, AtJAZ8m2 was strongly acetylated by HopZ1a.

(B) AtJAZ8m3 can still be acetylated by HopZ1a, in a similar strength as AtJAZ8. The aceyltation assays of AtJAZ8Δ1 and AtJAZ8m1 were repeated three times and the acetylation of AtJAZ8m2 and AtJAZ8m3 by HopZ1a were repeated twice with similar results.

A


AtJAZ8
AtJAZ8Δ1
AtJAZ8m1
AtJAZ8m2
Z1a
Z1a(C216A)
Z1a
Z1a(C216A)
Z1a
Z1a(C216A)
Z1a
Z1a(C216A)
Autoradiograph
AtJAZ8
HopZ1a
Coomassie blue staining
AtJAZ8
HopZ1a

B

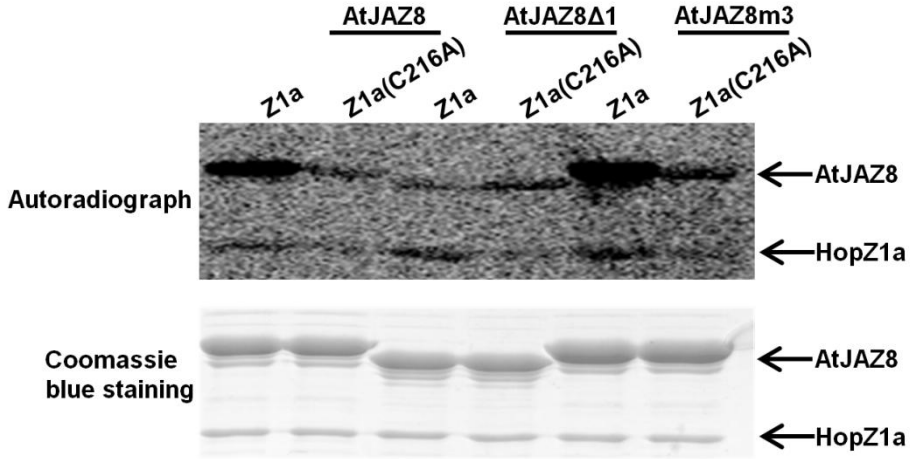
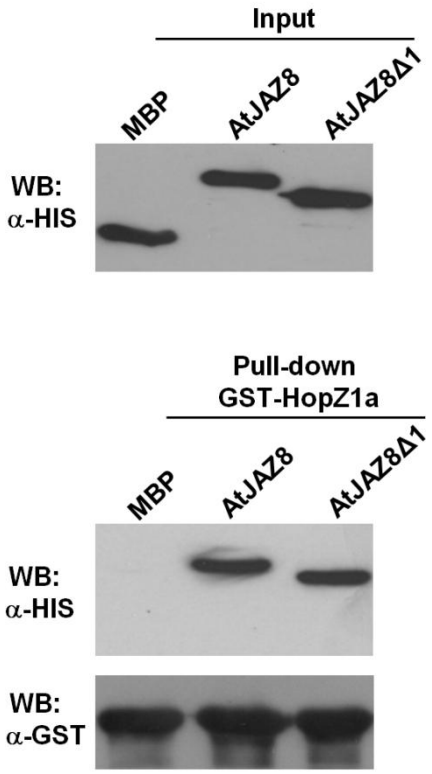

AtJAZ8
AtJAZ8Δ1
AtJAZ8m3
Z1a
Z1a(C216A)
Z1a
Z1a(C216A)
Z1a
Z1a(C216A)
Autoradiograph
AtJAZ8
HopZ1a
Coomassie blue staining
AtJAZ8
HopZ1a

Figure 2.6. AtJAZ8Δ1 interacts with HopZ1a in vitro.

GST-HopZ1a and MBP-AtJAZ8Δ1-HIS were expressed in *E. coli* strain BL21. Co-precipitation of AtJAZ8Δ1 with HopZ1a was determined by western blots before (Input) and after affinity purification (Pull-down) using anti-HIS antibody. The protein abundances of AtJAZ8 and AtJAZ8Δ1on the affinity resins after washes were detected by western blots using anti-HIS antibody.

This experiment was repeated three times with similar results.



HopZ1a does not degrade AtJAZ8 in N. benthamiana

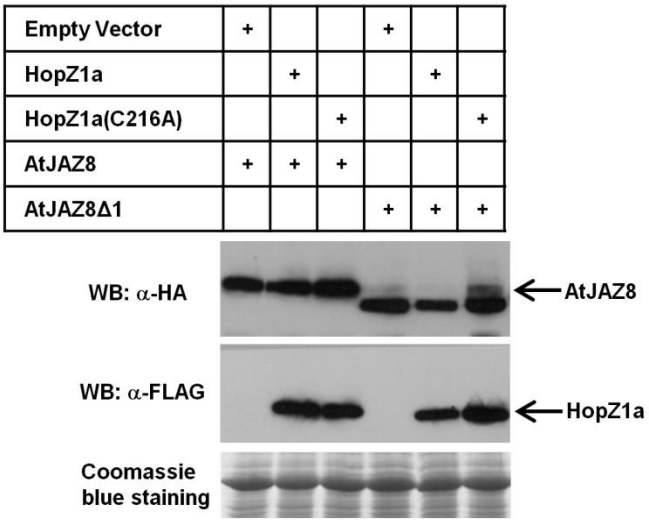
We previously observed that HopZ1a triggered the degradation of its soybean target proteins GmHID1 and GmJAZ1, as well as its *Arabidopsis* target AtJAZ6 when these proteins were co-expressed with HopZ1a in *N. benthamiana* (Jiang et al, 2013; Zhou et al, 2011). Therefore, I tested whether HopZ1a can mediate the degradation of AtJAZ8 using the same transient expression assay.

AtJAZ8 or its mutant AtJAZ8Δ1 were co-expressed with HopZ1a or its catalytic mutant HopZ1a(C216A) in the leaves of 4-week-old *N. benthamiana* plants. The total proteins were extracted at 20 hours post inoculation (dpi), which is usually ten hours earlier than the onset of detectable HopZ1a-caused cell death. Western blots were then used to determine the protein abundances. Unlike the other HopZ1 targets, the protein level of AtJAZ8 and AtJAZ8Δ1 remained the same in leaves co-expressing HopZ1a or HopZ1a(C216A) (Figure 2.7). These data demonstrate that HopZ1a is unable to induce the degradation of AtJAZ8 in *N. benthamiana*. Therefore, this experiment does not allow us to make a link between acetylation and the degradation of other HopZ1a targets, such as JAZ6.

Figure 2.7. AtJAZ8 cannot be degraded by HopZ1a when they are co-expressed in *N. benthamiana*.

YFP-AtJAZ8-HA and 3×FLAG-HopZ1a were transiently expressed in *N. benthamiana* and the abundances of AtJAZ8 or AtJAZ8Δ1 were detected by western blots at 20 hpi. The same protein gel was stained with Coomassie blue to show equal loading.

This experiment was repeated five times with similar results.



DISCUSSION

There are a total of twelve JAZ orthologs in *Arabidopsis*. Seven AtJAZs were previously tested for their association with HopZ1a and five were shown to interact with HopZ1a in vitro. In this study, in vitro acetylation assays showed that in addition to AtJAZ6, HopZ1a acetylates AtJAZ8, AtJAZ9, and AtJAZ10. No acetylation was observed using AtJAZ2, AtJAZ5, AtJAZ7, AtJAZ11 and AtJAZ12 as substrates under the same experimental condition. These data suggest that there are ample differences among AtJAZs in terms of their binding affinity and acetylation strength by HopZ1a. Of course, we could not exclude the possibility that the interaction/acetylation patterns of AtJAZs by HopZ1a may change under other in vitro conditions or in vivo. Indeed, I have observed some AtJAZs display drastically different binding activities with HopZ1a under different in vitro conditions. Furthermore, the in vitro acetylation assay may not be sensitive enough to detect inconspicuous acetylations. Unfortunately, there are no antibodies available to detect serine and threonine acetylation in vivo. Therefore, whether HopZ1a is able to acetylate more AtJAZs in vivo remains a difficult task to be addressed.

It is also possible that HopZ1a selectively acetylates a small portion of AtJAZs and that is sufficient for its virulence activity to promote infection. This raises interesting questions regarding to the functional redundancy and differentiation of JAZs. Except for the *AtJAZ10* loss-of-function mutants and an RNA-interference (RNAi) line of *AtJAZ1*, most *AtJAZ* mutants reported to date do not exhibit significantly enhanced sensitivity to JA treatment (Demianski et al, 2012; Grunewald et al, 2009; Yan et al, 2007). Besides, only the *AtJAZ10* mutants or the *AtJAZ10*

RNAi lines were reported to show enhanced susceptibility to *Pto*DC3118 (Demianski et al, 2012). In this regard, HopZ1a could be used as a tool to start dissecting JAZ functions in plant immunity. In addition, it is also likely that besides HopZ1a, more virulence factors can target different sets of JAZ proteins to benefit pathogen infections.

HopZ1a has been shown to acetylate AtJAZ6 and tubulin in *Arabidopsis* (Jiang et al, 2013; Lee et al, 2012). Here, I showed that HopZ1a also acetylates AtJAZ8, AtJAZ9, and AtJAZ10. Furthermore, a peptide TSYDSDSSDTTS that is close to the N-terminal EAR motif is critical for the acetylation of AtJAZ8 by HopZ1a. This peptide contains eight serine/threonine residues, which are candidate acetylation sites. Several YopJ-like effectors with acetyltransferase activity acetylate serine/threonine residues in their host targets (Mittal et al, 2006; Mukherjee et al, 2006; Paquette et al, 2012) (Trosky et al, 2007; Trosky et al, 2004). These data suggest that serines and threonines are common acetylation sites among the host targets of YopJ family effectors.

Mass spectrometry analyses revealed Thr^{25} as well as at least one additional Ser/Thr in the TSYDSDSSDTTS peptide of AtJAZ8 as potential acetylation sites by HopZ1a. The replacement of Thr^{25} by alanine did not influence the acetylation of AtJAZ8, consistent with the mass spec data that more than one amino acid can be acetylated. Future acetylation analysis using single/double/triple mutants of all the Ser/Thr sites within this peptide is required to unravel the exact acetylation sites of AtJAZ8.

AtJAZ8 could not be degraded by HopZ1a when they were co-expressed in *N. benthamiana*. However, we previously found that HopZ1a triggered the degradation of GmJAZ1, as well as AtJAZ6, using the same experiment (Jiang et al, 2013). These data suggest that AtJAZ8 is distinct from these other JAZs. A recent publication characterized AtJAZ8 and showed that AtJAZ8 could not bind with COI1 in the presence of JA-Ile in the in vitro assay and was resistant to JA-mediated degradation in vivo (Shyu et al, 2012). Domain swap experiments revealed that AtJAZ8 lacks a canonical JA-Ile degron which is required for JA-Ile-dependant COI1-AtJAZ8 interaction. However, high concentration of coronatine was still able to induce weak association of AtJAZ8 and COI1 and thereby leading to the turnover of AtJAZ8 (Shyu et al, 2012). Importantly, this AtJAZ8 degradation can be partially prohibited by the pretreatment of *Arabidopsis* seedlings with the 26S protease inhibitor MG132 (Shyu et al, 2012). These data suggest that AtJAZ8 could be degraded in a pathway involving COI1 and 26S proteasome, but this degradation might require potent ligands, longer time, or both. Compared to coronatine, HopZ1a only leads to partial degradation of AtJAZ1 in *Arabidopsis* and partially complements the growth deficiency of the coronatine mutant *Pto*DC3118. It is possible that the degradation of AtJAZ8 by HopZ1a needs a longer period of time, and the relatively short co-expression time of AtJAZ8 and HopZ1a in *N. benthamiana* might be not enough for us to observe AtJAZ8 degradation. To address this issue, I have started the process to generate *AtJAZ8* over-expression lines in *Arabidopsis zar1-1* background in order to test whether HopZ1a can reduce the abundance of AtJAZ8 during bacterial infection.

AtJAZ8Δ1 and *AtJAZ8m1* transgenic lines are also being prepared to investigate potential correlations between acetylation and degradation of AtJAZ8 by HopZ1a.

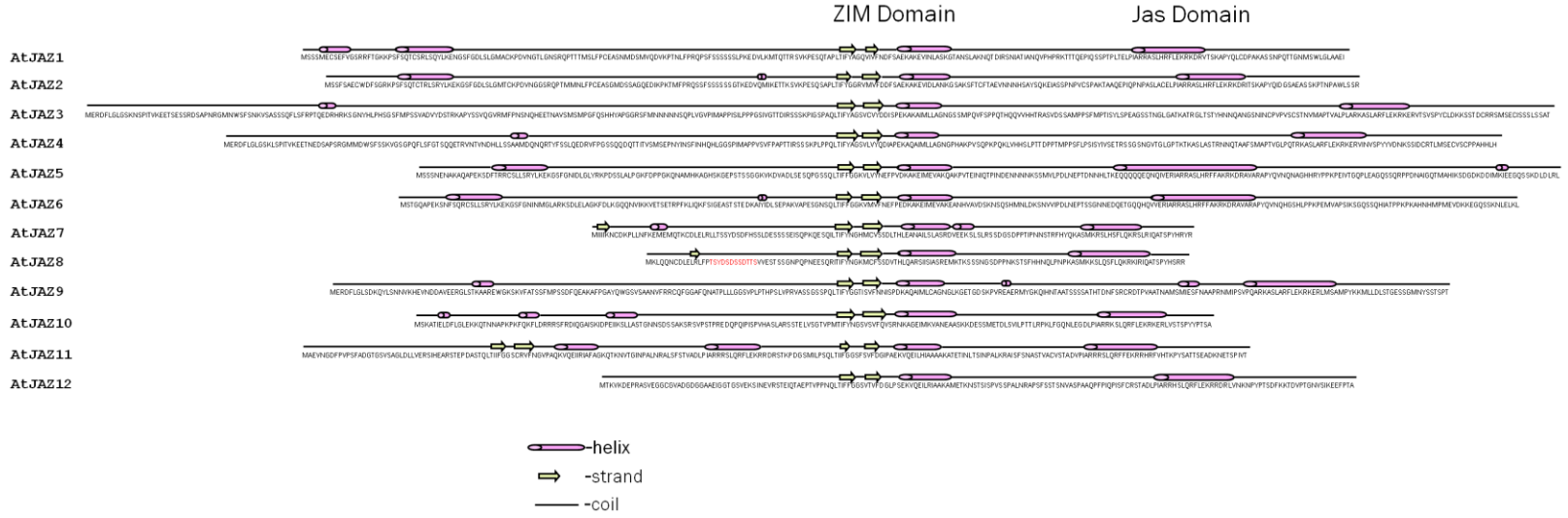
Different from other AtJAZs, AtJAZ8 employs a distinct mechanism to repress the transcription of JA-responsive genes. The N-terminal LxLxL-type EAR motif of AtJAZ8 directly recruited the co-repressor TOPLESS and repressed the transcription activation of JA signaling (Shyu et al, 2012). The TSYDSDSSDTTS region that is essential for AtJAZ8 acetylation by HopZ1a is at the immediate proximity of the EAR motif. It is tempting to speculate that the acetylation of this peptide might also be able to modulate the function of AtJAZ8 by interfering with the interaction between the AtJAZ8 EAR motif and the TPL co-repressor. Further experiments testing this possibility will help reveal novel regulatory mechanisms underlying the functionality of JAZs.

According to our in vitro acetylation results, AtJAZ6 and AtJAZ10 were also acetylated by HopZ1a. However, whether HopZ1a can induce/promote the destabilization of AtJAZ10 has not been examined. Furthermore, because the TSYDSDSSDTTS sequence of AtJAZ8 is not conserved in AtJAZ6 or AtJAZ10, it is necessary to also determine the acetylation sites in these two AtJAZ proteins using mass spectrometry. Comparing the acetylation sites of AtJAZ6, AtJAZ8 and AtJAZ10 will then provide mechanistic insights into the enzymatic activity of HopZ1a. One possibility is that specific amino acids surrounding certain secondary structure of AtJAZs are involved in their acetylation by HopZ1a. I performed a secondary structure analyses of twelve AtJAZs using the software PSIPRED(Figure 2.8) (McGuffin et al, 2000). This analysis showed a conserved structure consisting of two

strands following by an α-helix, which is located in the ZIM domain among all the AtJAZs. Interestly, the acetylated region in the AtJAZ8 is in the immediate upstream of this conserved secondary structure. Indeed, many JAZs have regions enriched with Ser/Thr in the corresponding locations. Further experimens are needed to examine whether these Ser(s)/Thr(s) are important for the JAZ acetylation.

Figure 2.8. Secondary structure analyses of AtJAZs using PSIPRED.

The peptide TSYDSDSSDTTS in AtJAZ8 that contains acetylation site(s) is highlighted in red. The secondary structures for each AtJAZ are listed on top of the corresponding protein sequences. The pink cylinders represent helixes, the yellow arrows represent strands and the black lines represent the coils.


ZIM Domain
Jas Domain
AtJAZ1
AtJAZ2
AtJAZ3
AtJAZ4
AtJAZ5
AtJAZ6
AtJAZ7
AtJAZ8
AtJAZ9
AtJAZ10
AtJAZ11
AtJAZ12
-helix
-strand
-coil

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