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Biochemical and Functional Characterization of the *Pseudomonas syringae* Effector HopZ1a

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by

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Figure viii in the General Introduction is a reprint of materials as it appears in Ma, K.-W., Flores, C., and Ma, W. (2011). Chromatin Configuration as a Battlefield in Plant-Bacteria Interactions. *Plant Physiology.* 157, 535-543.

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Ka-Wai Ma, and Dr. Wenbo Ma conceived, designed the experiments, and wrote the paper.
Dr. Shushu Jiang performed biological experiments as shown in Figures 2 and generated some plant material and plasmid constructs.
Dr. DongHyuk Lee and Dr. Gitta Coaker repeated the mass spectrometry analysis for Figure 1b.
Dr. Songqin Pan participated in the design and analysis of data for Figure 1b. Dr. Jikui Song provided technical expertise in protein purification and the NMR study.
Dr. Dan Borchardt provided technical assistance for the NMR study.

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Pursuing a doctoral degree in a foreign country is one of the biggest decisions I have made in my life. Without any idea what challenges are waiting ahead for me, I was naïve enough to think that the graduate school was just an extension from my undergraduate life. And it is indeed not as simple as I understand now. I am very fortunate to have many mentors and teachers who guide me throughout my life. My foremost gratitude goes to my PhD supervisor Dr. Wenbo Ma, for not just providing guidance and insights for my projects, but also serving as a role model showing me the enthusiasm and meticulous scholarship every scientist should be proud of. Without her continuous supervision and also occasional laissez-faire style of management that gives me freedom to explore various possibilities, this dissertation would not be possible.

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ABSTRACT OF THE DISSERTATION

Biochemical and Functional Characterization of the *Pseudomonas syringae* Effector HopZ1a

by

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Doctor of Philosophy, Graduate Program in Plant Pathology
University of California, Riverside, August 2015
Dr. Wenbo Ma, Chairperson

Gram negative pathogens rely on the Type III secretion system to inject virulence proteins (aka secrete effectors) into eukaryotic hosts to manipulate defense. YopJ is one of the largest effector family prevalently produced by both animal and plant pathogens. HopZ1a from the plant pathogen *Pseudomonas syringae pv. syringae* A2 possesses an acetyltransferase activity and acetylates target proteins in plant hosts for virulence.

In chapter I, I used biochemical approaches to characterize the residues required for the enzymatic activity of HopZ1a. I identified a threonine residue T346 as the autoacetylation site of HopZ1a. I also found two serine residues S349 and S351 required for the avirulence and virulence function of HopZ1a, through their contributions to the acetyltransferase activity. The acetyltransferase activity of HopZ1a can be activated by the eukaryotic cofactor inositol hexakisphosphate (IP6). My experiments showed that S349 and S351, together with a previously identified lysine residue K289, were essential

to mediate IP6-induced conformational change of HopZ1a. The conservation of S349 and S351 and the requirement of them in the acetyltransferase activity of other YopJ family effectors suggest that additional residues in addition to the indispensable catalytic triad are required for the biological function of HopZ1a.

In chapter II, I used traditional genetics and molecular biology approaches to study the function of an interacting partner of HopZ1a in soybean called GmZINP2. By using transgenic soybeans over-expressing GmZINP2, I showed that GmZINIP2 is a negative regulator of plant immunity and suppresses early and late defense responses. GmZINP2 contains domains that are likely associated with chromatin remodeling functions. I showed that GmZINP2 could bind DNA and also interacts with a transcription factor GmMYB56 in soybean. Interestingly, HopZ1a could acetylate GmZINP2 and affect the interaction between GmZINP2 and GmMYB56. As such, HopZ1a may manipulate soybean defense through its interaction with GmZINP2.

This thesis represents a comprehensive study on the enzymatic activity and virulence functions of an important class of type III effectors. The study on GmZINP2 revealed possible manipulation of host defense by bacterial pathogens through chromatin remodeling process, which is a novel strategy utilized by bacterial pathogens to enhance virulence.

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GENERAL INTRODUCTION

Plant-microbe interactions

Plants are sessile organisms that are constantly interacting with other microbes. The interactions between them are extremely diverse: saprophytic microbes like soil born bacteria *Bacillus cereus* and *Pseudomonas fluorescens* gain nutrients from dead organic materials without directly impacting the living plants (Vilas-Boas et al., 2007; Mithani et al., 2011; Inacio et al., 2015); in the case of mutualistic interactions, both plants and microbes benefit from each other as exemplified by the nitrogen fixing rhizobia and legumes (Udvardi and Poole, 2013). Finally, a small group of microbes are evolved to be pathogenic, where they cause damage to the plant hosts for their own benefit.

Plant infecting pathogens are classified into two classes: pathogens like *Erwinia carotovora* produces a number of cell wall degrading enzymes and kills cells to feed on dead materials for nutrients and they are collectively known as necrotrophs (Toth and Birch, 2005); on the other hand, pathogens including the downy mildew oomycete *Hyaloperonospora parasitica* (*Hpa*) are obligate biotrophs that only survive on living hosts (Coates and Beynon, 2010). Most pathogens including the bacterial blight bacterium *Pseudomonas syringae* can adopt a more flexible infection strategy as a hemibiotroph, shifting from a biotroph to a necrotroph as disease progresses (Xin and He, 2013). However, the aforementioned plant-microbe interactions can be dynamic and change over time. For instances, saprophytic *B. cereus* can also colonize invertebrate gut and causes severe diarrhea (Jensen et al., 2003); *P. syringae* mostly exists as an epiphyte

without causing obvious symptoms on the host and only becomes a devastating pathogen when the population reaches a threshold as sensed by the density-dependent quorum sensing system, resulting in the activation of different pathogenesis related genes (Chatterjee et al., 2007).

Overview of the plant innate immunity system and plant-pathogen arms-race

As plants are constantly challenged by various potential pathogens, the ability to respond in a timely and efficient manner is crucial for their survival. Unlike animal that contains an adaptive immunity system that can fight against different pathogens, plants depend on the recognition of non-self and activation of innate immunity as their major defense response (Maekawa et al., 2011). The plant innate immune system is divided into two branches: pattern-triggered immunity (PTI) and effector-triggered immunity (ETI) (Jones and Dangl, 2006; Spoel and Dong, 2012).

The first branch of plant immunity is based on the activation of PTI upon recognition of evolutionarily conserved microbial signatures called microbe- or pathogen-associated molecular patterns (MAMPs or PAMPs) by pattern recognition receptors (PRRs) located on the plasma membrane (Bohm et al., 2014; Zipfel, 2014). flg22, the conserved N-terminal 22-amino acid epitope from *P. syringae* flagellin, is one the best studied PAMPs (Felix et al., 1999) (Figure i). Activation of flg22-triggered signaling depends on FLS2 and the co-receptor BAK1 (Gomez-Gomez and Boller, 2000; Chinchilla et al., 2007). Co-crystallization of the leucine rich repeat domain of FLS2 and BAK1 with flg22 revealed that flg22 serves as a molecular glue to bring FLS2 and BAK1 together, resulting in the subsequent phosphorylation events and signal transduction (Sun

et al., 2013). Following flg22 binding, FLS2 activation is attenuated by BAK1-dependent phosphorylation and recruitment of two E3 ligases PUB12 and PUB13 to the FLS2 complex. PUB12 and PUB13 ubiquitinate FLS2 and promotes its degradation (Lu et al., 2011). In addition, PUB12/13 mediated-FLS2 degradation is independent of the endocytosis-dependent FLS2 degradation pathway (Robatzek, 2006), suggesting that FLS2 stability can be regulated through different mechanisms.

Other examples of PAMP-PRR pairs include the conserved N-terminal 18-amino acid epitope (elf18) from bacterial elongation factor Tu (EF-Tu) and the EF-Tu RECEPTOR (EFR) (Zipfel et al., 2006); chitin from the cell wall of fungi and the rice CERK1 (Miya et al., 2007). Even though different PRRs are closely related, they can be specific to certain plant species. For instances, EFR is only found in *Brassicaceae* family (Kunze et al., 2004; Zipfel et al., 2006) and the rice receptor XA21 recognizing *Xanthomonas oryzae* is limited in the rice species *Oryza longistaminata* (Song et al., 1995; Song et al., 1997). Interestingly, successful transformation of a chimeric combination of the ectodomain from EFR to the transmembrane and intracellular domain of XA21 enables rice to recognize elf18, raising the possibility to engineer chimeric PRRs across species to enhance disease resistance (Schwessinger et al., 2015).

Ligand-induced PRR activation leads to a series of physiological responses from production of reactive oxygen species (ROS), activation of Ca^{2+} dependent protein kinases (CDPK), and mitogen activated protein kinases (MPKs) signaling, followed by the production of antimicrobial compounds, fortification of cell wall with callose, restriction of bacterial entry through stomatal closure and amplification of defense

response through phytohormone pathways (Zipfel, 2009; Bigeard et al., 2015). Although PTI effectively prevents infection from a majority of potential pathogens, it can be defeated by pathogens that have evolved different virulence factors. For examples, Gram-negative bacteria use the type III secretion system (T3SS) to deliver type III secreted effectors (T3SEs) into host cells (Galan et al., 2014). Once inside their hosts, T3SEs directly and indirectly interfere with targets contributing to plant immunity, and thereby promoting pathogen entry, colonization and disease development (Feng and Zhou, 2012). Such a T3SE-dependent suppression of immunity is called effector-triggered susceptibility (ETS). Similar deployment of effectors can be observed in fungi and oomycetes through haustoria or herbivores through physical injection. As this dissertation focuses on the function of the bacterial T3SE HopZ1a, effectors produced by eukaryotic pathogens will not be discussed in details. Readers are directed to several articles that systemically reviewed these topics, such as (Torto-Alalibo et al., 2009; Wawra et al., 2012).

The second branch of plant immunity depends on resistance (R) proteins, which were evolved as a counterattack strategy to recognize effector activities (Jacob et al., 2013; Qi and Innes, 2013). Nucleotide-binding and leucine-rich repeat (NB-LRR) proteins are the major class of R protein. The N-terminus of NB-LRR protein can be associated with either the coiled coil domain (CC) or toll and human interleukin-1 receptor (TIR) domain (Dangl and Jones, 2001; Lukasik and Takken, 2009) (Figure iiA). Activation of R proteins leads to robust effector-triggered immunity (ETI) that usually involves the development of programmed cell death at the infection sites, called the

hypersensitive response (HR). Because of this, effectors were referred as avirulence proteins when they trigger HR in plants producing cognate R proteins. Since the introduction of the gene-for-gene concept by Flor back in the 1940s, dozens of Avr-R pairs have been uncovered. Even though direct interactions between an effector and a R protein has been demonstrated, e.g. AvrPita and Pita (Jia et al., 2000), AvrL567 and L (Dodds et al., 2006), and PopP2 and RRS1-R (Deslandes et al., 2003), most effectors are recognized indirectly. The guard hypothesis was originally proposed to account for the interaction between AvrPto and Pto, where Pto is "guarded" by a canonical NB-LRR protein Prf (Salmeron et al., 1994; Salmeron et al., 1996; Oldroyd and Staskawicz, 1998). The central idea of the "guard model" is based on the assumption that the guarded protein (guardee) is required for effector virulence function (van der Biezen and Jones, 1998) (Figure iiB). However the guard model cannot account fully for the opposing selective forces existing in a population such that the presence of R protein selects for increasing effector-guardee interaction to trigger ETI whereas the absence of R protein selects against effector-guardee interaction in favor of effector virulence. Furthermore, additional effector targets that do not seem to be required for immunity function were discovered. These lead to the proposition of another model called the decoy model (Hoorn and Kamoun, 2008). Unlike the guard model, the decoy model (Hoorn and Kamoun, 2008) (Figure iiB) suggests that the effector targets were evolved solely for the purpose of recognizing the presence of effectors without a direct involvement in establishing immunity.

Figure i. Physiological events during activation and attenuation of flg22 perception. Flg22 serves as a molecular glue to bring FLS2 and BAK1 together, which in turns *trans*-phosphorylates each other and BIK1. Phosphorylated BIK1 dissociates from the FLS2-BAK1 complex and initiates downstream signaling events. FLS2 activation leads to a variety of physiological events including the activation of MAPK signaling, Ca^{2+} influx, the activation of CDPK-dependent signaling, and ROS burst. Flg22-dependent signaling can be attenuated by two mechanisms: 1) FLS2 degradation dependent on the E3 ligases, PUB12 and PUB13, which are recruited to ubiquitinate FLS2 upon phosphorylation by BAK1; 2) FLS2 can also be degraded through endocytosis and the vacuolar pathway.

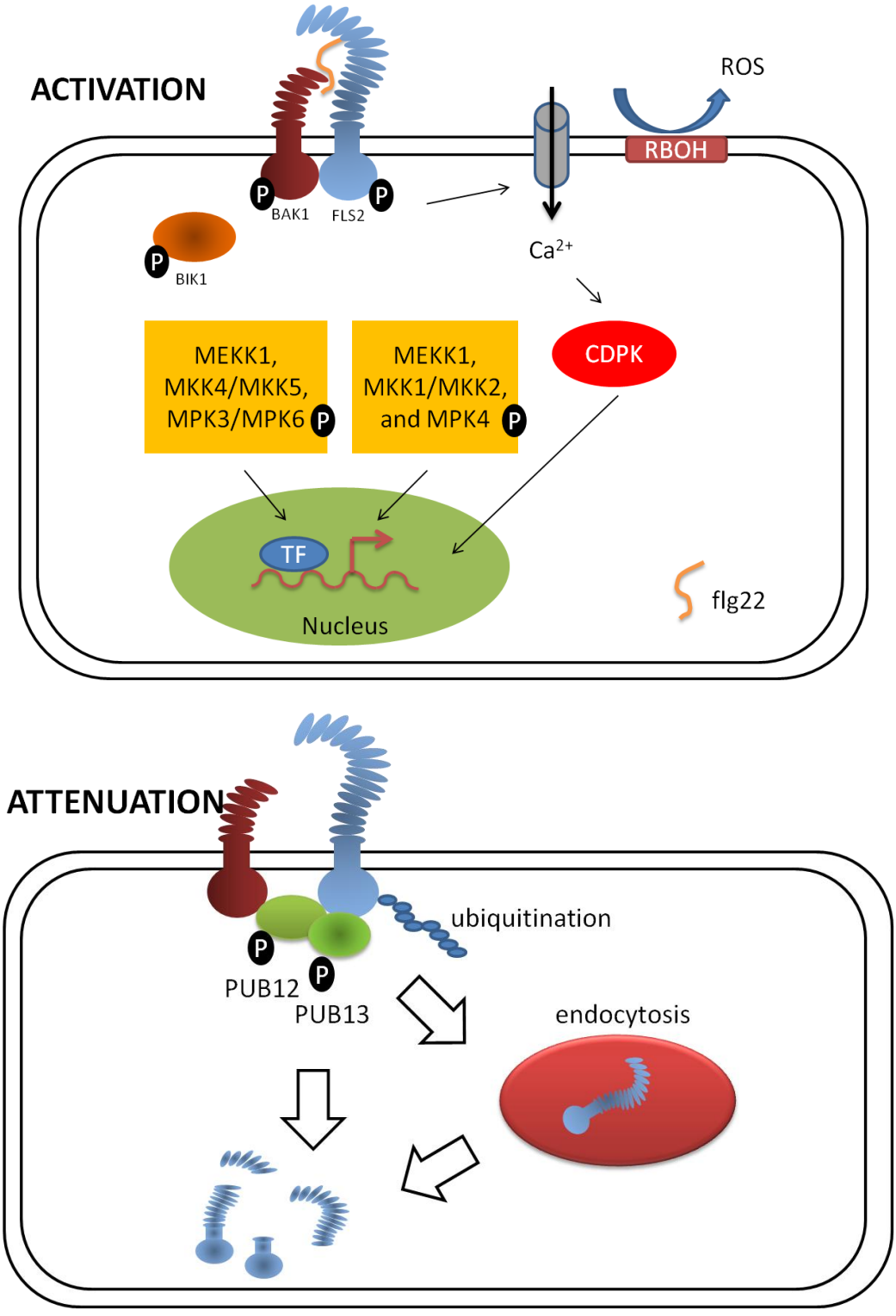
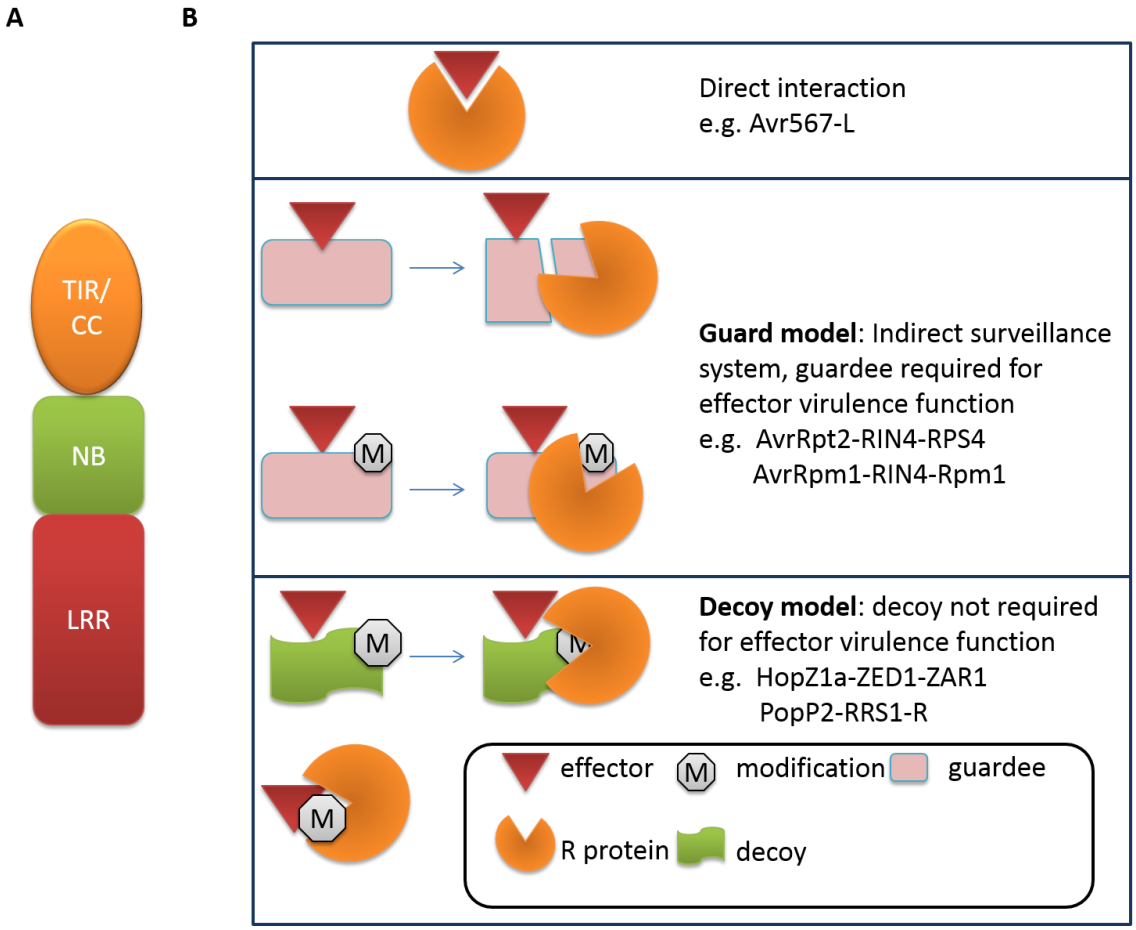

ACTIVATION
ROS
RBOH
P
BAK1
FLS2
P
P
BIK1
Ca2+
MEKK1,
MKK4/MKK5,
MPK3/MPK6
P
MEKK1,
MKK1/MKK2,
and MPK4
P
CDPK
TF
Nucleus
flg22
ATTENUATION
ubiquitination
P
P
PUB12
PUB13
endocytosis

Figure ii. Guard and decoy model of R-protein mediated recognition of effectors.

A,NB-LRR proteins can be divided into three regions: C-terminal leucine-rich repeat (LRR), nucleotide binding NTPase domain (NB), and N-terminal coiled coil (CC) or toll and human interleukin-1 receptor (TIR) domain. CC and TIR domains are not present in all NB-LRR proteins.

B, Schematic illustration of the guard and decoy model. The presence of effector can be recognized through direct interaction between effector and R-protein, or indirectly through the guardee or decoy.



Two recent studies on the T3SE PopP2 and the cognate R protein RRS1-R nicely demonstrated how a protein can serve as both a decoy and an R protein at the same time. PopP2 from *Ralstonia solanacearum* is an acetyltransferase that modifies WRKY transcription factors to promote infection (Le Roux et al., 2015; Sarris et al., 2015). WRKY transcription factors are involved in the regulation of the expression of defense related genes (Eulgem and Somssich, 2007; Buscaill and Rivas, 2014). Acetylation of WRKYs by PopP2 affects their DNA binding activity. In Arabidopsis eco. Nd-1, PopP2 can be recognized by RRS1-R, which carries a WRKY domain at the C-terminus. PopP2-mediated acetylation on a conserved lysine residue within the WRKY domain of RRS1-R inhibits its DNA binding activity, possibly through lowering of the electrostatic interactions between positively charged lysine and DNA, which in turns activates defense. Interestingly, PopP2 also acetylates another RRS1 allele RRS1-S and affects its DNA binding activity, but no HR is observed in PopP2-RRS1-S interaction (Le Roux et al., 2015; Sarris et al., 2015). Further study on the recognition specificity and acetylation-mediated inhibition of DNA binding activity will provide more insights on how R proteins may regulate defense following activation.

The activation of strong ETI in the presence of R proteins often masks the virulence function of effectors, rendering plants resistant to infection. In response to the presence of the corresponding R protein, pathogens may overcome ETI through: 1) loss of ETI triggering effectors; 2) evasion of ETI via effector polymorphism; and 3) acquisition of additional effectors to counteract ETI responses (Ma and Guttman, 2008). Such an evolutionary arms-race on effectors and R proteins is coined as the “zig-zag” model and

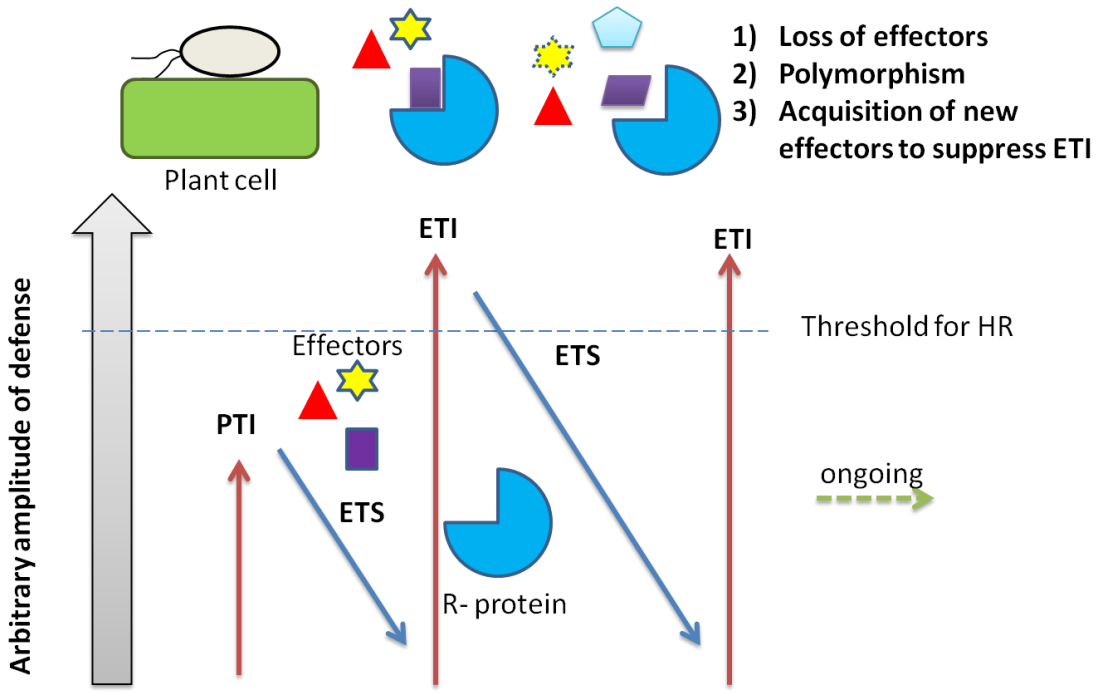
generally applies to different pathosystems (Jones and Dangl, 2006; Ma and Guttman, 2008) (Figure iii).

Loss of ETI-triggering effectors could be observed in the co-evolution between *P. syringae* and common bean. AvrPphB is a T3SE from *Pseudomonas syringae* pv. *Phaseolicola* (*Pph*) that is recognized by the R protein R3. In the presence of R3, the pathogenicity island harboring AvrPphB, PPHGI-1, could be excised and lost in a series of plant passage experiments, shifting the interaction from incompatible to compatible (Pitman et al., 2005). However the loss of effector can be concomitant with reduced pathogen fitness due to plant immunity; in this case, the second strategy would be more favorable. Evasion of ETI via effector polymorphism is experimentally demonstrated in the *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) type III effector AvrBs3 and its cognate R protein Bs3. The central region of AvrBs3 consists of tandem repeat units that determine its DNA binding specificity (Bochtler, 2012). AvrBs3 is recognized by the R protein Bs3 in pepper through binding to *Bs3* promoter and subsequent transcription activation of *Bs3*. Deletion of part of the tandem repeats of AvrBs3, i.e. AvrBs3Δrep16, abolished the induction of HR through Bs3. However plants turn the tide again in favor of them by evolving Bs3-E, an allele of Bs3 that regains the ability to recognize AvrBs3Δrep16 (Romer et al., 2007). These examples nicely demonstrate the evolutionary arms-race between plants and pathogens. The last strategy of counteracting ETI through acquisition of additional effectors is by far the most common strategy demonstrated (Jamir et al., 2004; Kang et al., 2004; Guo et al., 2009; Macho et al., 2010; Block et al., 2014; Teper et al., 2014), which is also partially refractory to the large number of effector

repertoire existing among different pathogens (Lindeberg et al., 2006; Birch et al., 2008; Bozkurt et al., 2012; Lindeberg et al., 2012). The virulence mechanisms of various T3SEs will be discussed in later sections.

Figure iii. Zig-zag model of host-pathogen arms race.

The zig-zag model depicts the evolutionary arms-race between pathogen and host (Adapted from Jones and Dangl, *Nature*. 2006). Perception of conserved molecular signature including flg22 and chitin activates the basal and broad spectrum PTI. Effectors are delivered to suppress PTI, resulting in ETS. Plants evolve R proteins to recognize effector presence directly or indirectly, resulting in the activation of robust ETI. Additional effectors may be employed to counteract ETI defense and the arm race continues as new effectors and R protein are evolved.



Integration of environmental signals into plant innate immunity

The disease triangle proposed by Steven back in the 1960s is the central paradigm in plant pathology. It states that the result of a disease from a biotic agent consists of at least three components: the susceptible host, the virulent pathogen and the environment. Over the years, extensive effort has been made in elucidating the relationships between the host and the pathogen and the underlying mechanisms. Recently, attention has also been diverged to the effect of environmental factors in a pathosystem (Hua, 2013).

Temperature is one of the abiotic factors that impose significant impact on the outcome of pathogenesis. It was demonstrated in Arabidopsis that low ambient temperature i.e.10–23 °C preferentially activates ETI response and high temperature activates PTI responses. And such a modulation of defense response is dependent on histone modification (Cheng et al., 2013). The enhancement of PTI at higher temperatures is likely an adaptation of plants against accelerated bacterial proliferation. It is not well understood why ETI is inhibited with increasing temperature. On one hand, it was known that the T3SS and the secretion of several effectors were promoted at low temperature (van Dijk et al., 1999), which matches with the enhanced ETI responses at such condition. However the effects of temperature in ETI against different pathogens are sometimes contradictory and should be considered in a case specific manner (Hua, 2013). The discovery of various temperature sensitive R gene mutants further highlights the complexity of temperature in ETI responses (Wang et al., 2009; Zhu et al., 2010; Mang et al., 2012).

Light and the circadian clock are other factors that are involved in the regulation of plant innate immunity as evidenced by the altered defense response in different photoreceptor mutants (Griebel and Zeier, 2008; Wu and Yang, 2010; Xie et al., 2011). A comprehensive analysis of the promoters of different defense associated genes during *Hpa* infection identifies conserved sequence known as the "evening element", which is regulated by the circadian regulator CCA1. The rhythmic expression pattern of these genes without pathogen infections suggests that they are programmed to anticipate specific events. Peak expression of these genes at night coincides with the time of *Hpa* sporulation; and inoculation of pathogens at unexpected time i.e. dusk, substantially enhanced susceptibility (Wang et al., 2011), further confirming the role of circadian clock in basal defense response. Circadian clock regulated defense can be exhibited as stomata dependent and independent responses. It is proposed that plants rely more on stomata closure to restrict pathogen invasion at night, whereas stomata-independent defense is more prominent at daytime when stomata have to be open for photosynthesis in sacrifice of enhanced pathogen access. Furthermore, pathogen infections can feedback to the circadian clock, providing an additional level of regulation to fine-tune defense responses (Zhang et al., 2013). More recently, non-transcriptional rhythm of redox has been demonstrated in plants (Edgar et al., 2012). As redox status has long been shown to potentiate the function of a key defense regulator, NPR1 (Mou et al., 2003), the redox circadian clock thus regulate plant defense in a NPR1-dependent manner (Zhou et al., 2015a).

Bacterial toxins

Bacterial toxins are among the best studied examples of virulence factors next to T3SEs (Bender et al., 1999). Syringolin A (SylA), coronatine (COR) and rhizobitoxine are three toxins that interfere with the phytohormone signaling pathways for virulence. Phytohormone signaling regulated by salicylic acid (SA), jasmonates (JA) and ethylene (ET) are major defense hormone pathways against biotrophs and necrotrophs respectively (Glazebrook, 2005). SylA is a peptide derivative synthesized by *P. syringae* pv. *syringae* B728A. SylA irreversibly inhibits the function of proteasomes through binding to the catalytic sites. Since proteasome regulates the stability of NPR1, the key regulator of SA-mediated defense, inhibition of proteasome leads to improper NPR1 turnover and compromised SA-dependent immunity (Groll et al., 2008; Schellenberg et al., 2010; Misas-Villamil et al., 2013). Coronatine (COR) is a multifaceted toxin produced by *P. syringae* pv. *tomato* DC3000 (*Pto*DC3000) as well as a few other *Pseudomonas syringae* strains through manipulation of phytohormone signaling. JA signaling is activated through the degradation and subsequent depression from the negative regulator, Jasmonate Zim-domain proteins (JAZs), in a Coronatine Insensitive 1 (COI1) dependent manner (Thines et al., 2007; Melotto et al., 2008; Sheard et al., 2010). COR up-regulates JA signaling by structurally mimicking the active forms of JA, which promotes JAZ degradation (Feys et al., 1994; Weiler et al., 1994; Katsir et al., 2008). As SA and JA signaling are antagonistic to each other, COR suppresses defense against *Pto*DC3000 through indirect suppression of SA pathways. In addition, COR suppresses SA accumulation by inducing the expression of three NAC transcription factors, ANAC019,

ANAC055 and ANAC072 (Zheng et al., 2012). During natural infection, COR manifests its virulence function by reopening of closed stomata in response to pathogen perception (Melotto et al., 2006). COR also suppresses the biosynthesis and subsequent deposition of callose, however the underlying mechanism is not fully understood (Geng et al., 2012). Rhizobitoxine is produced by *Burkholderia andropogonis* and several *Bradyrhizobium* spp.. Rhizobitoxine is considered as a toxin because it induces chlorosis through inhibition of ethylene biosynthesis (Owens et al., 1971). Rhizobitoxine has also been shown to regulate the establishment of symbiosis between rhizobia and legumes (Ruan and Peters, 1992), indicating the diverse roles of this toxin in plant-microbe interactions.

Syringomycin and syringopeptin are lipopeptide toxins that target the plasma membrane for virulence. These toxins are characterized with a hydrophilic peptide head and hydrophobic fatty acid tails. Such an amphipathic property allows these toxins to be inserted into the plasma membrane and form pores in plant cells, facilitating leakage of nutrients and potential alkalization of the surrounding environment to favor bacterial survival (Iacobellis et al., 1992; Hutchison et al., 1995).

Tabtoxin and phasolotoxin are toxins that induce chlorosis by targeting glutamine synthetase and ornithine carbamoyl transferase respectively (Mitchell and Bieleski, 1977; Thomas et al., 1983). So far, the biological significance of these toxins remains elusive.

Type III secretion system (T3SS)

Apart from producing toxins, Gram negative bacteria depend on the T3SS to deliver effectors into eukaryotic cells to suppress defense response. Originally identified from *Salmonella typhimurium*, cryo-electron microscopic study on other bacteria revealed

extensive similarity of the system among different bacterial species. In general, the secretion apparatus or injectisome can be divided into three parts: the base, the needle and the translocon (Figure 4). The base consists of ring like structures embedded in the bacterial envelope together with different cytosolic proteins. The base is connected to a needle-like extrusion assembled from numerous subunits in a helical form. Finally the assembly of the complex is completed by the translocon connected by the tip protein. The translocon presumably inserts itself into the host membrane to form a channel for the secretion of effectors into the host. Together, the syringe-like apparatus acts as nano-machine to actively secrete effectors into eukaryotic cells (Pallen et al., 2005; Cornelis, 2006; Galan et al., 2014).

The exact mechanism of how effectors are recognized and secreted by the T3SS is not well understood. It is proposed that the recognition specificity of cognate effectors is governed by loosely defined secretion signal often found in their N-terminus. Additional sequences may serve as binding sites to chaperones, which work together with ATPases to unfold effector protein before going through the injectisome. The energy stored in the unfolded protein can then be used to drive the passage of effectors through the channel, although other energy source including proton motive force (PMF) has also been suggested (Cornelis, 2006; Galan et al., 2014).

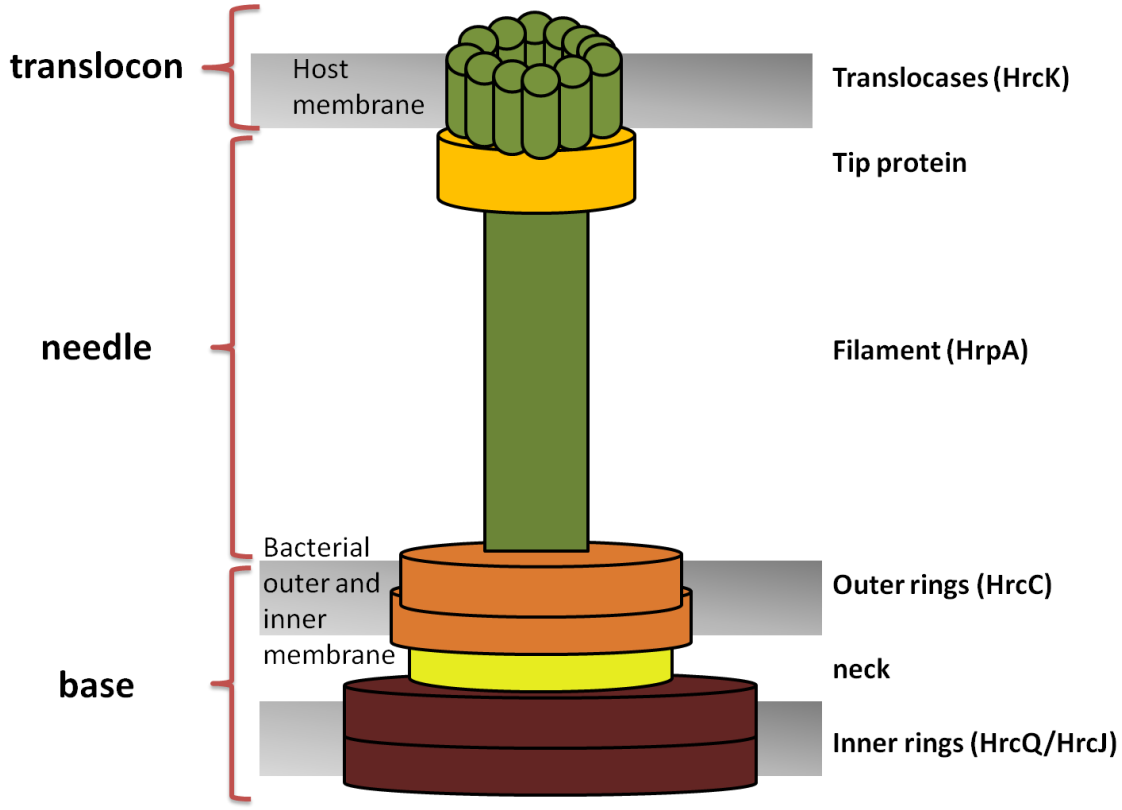
The T3SS in plant pathogens are encoded by genes known as hypersensitive response and pathogenicity (*hrp*) genes, which are often clustered in big pathogenicity islands (Bogdanove et al., 1996). Induction of the T3SS during pathogen infection has been known for a long time, but it is not until recently the underlying mechanism is

starting to unveil. Using a metabolomic approach comparing the plant exudates from wild type and *mkp1*, a mutant in which delivery of T3SEs is suppressed, it was shown that different plant metabolites including citric acid, aspartatic acid and shikimic acid significantly enhance T3SS expression and delivery of T3SEs (Anderson et al., 2014). In addition, metabolites such as citrate, was also previously shown to be a strong chemo-attractant (Cuppels, 1988) and inducer of phytotoxin coronatine production (Li et al., 1998), indicating a general role of these metabolites in activating different virulence mechanisms. However, how MKP1 regulates these metabolites in exudates and the receptor(s) for these compounds in bacteria are still elusive.

Even though the roles of the T3SS in pathogenesis have been well documented (Hueck, 1998; Collmer et al., 2000), the widespread distribution of the T3SS in different bacteria from pathogens to symbionts suggests that T3SS is involved in general host-microbe interaction but not limited to pathogenesis (Galan et al., 2014). A recent survey of the natural population of *P. syringae* also identified strains that lacked a canonical T3SS but still able to cause disease symptoms in cantaloupe seedlings, raising questions about the dispensability of the T3SS in pathogenesis (Diallo et al., 2012). However, it is still possible that the observed disease symptoms may be attributed to an unknown/un-typical T3SS present in these strains. Until then, T3SS should still be considered as an essential component in phytopathogen pathogenesis.

Figure iv. Type III secretion system (T3SS) in Gram negative bacteria.

General organization of the T3SS in Gram negative bacteria. The secretion apparatus is divided into three parts: the base, the needle and the translocon. Different components involved in the assembly of the injectisome as named in *Pseudomonas syringae* are given on the right.



Type III secreted effector (T3SE) repertoire

As discussed in the previous section, T3SS is essential for bacterial pathogenesis due to its ability to deliver T3SEs into eukaryotic host cells. Effectors are proteins that modulate the host structure and physiology. The ultimate function of these T3SEs is to suppress defense response; regardless it is PTI or ETI. Around 60 T3SEs have been identified in *P. syringae* and they contribute significantly to pathogenesis by different mechanisms suppressing plant defense response (Boller & He, 2009).

Most of our knowledge on bacterial T3SEs came from studies on the model organism *P. syringae* during infection of several plant species, especially Arabidopsis. *P. syringae* species consists of around 50 pathovars with variable host ranges (Sawada et al., 1999; Sarkar and Guttman, 2004). The publication of the full genome sequence of *Pto*DC3000 back in 2003 (Buell et al., 2003), followed by several other *P. syringae* pathovars (Feil et al., 2005; Joardar et al., 2005) and the use of a differential fluorescence induction (DFI) and fluorescence-activated cell sorter approach enabled the discovery of near-complete repertoires of T3SEs in these strains (Chang et al., 2005). Currently around 80 T3SEs are named for *P. syringae* and only a few of them are conserved among *Pto*DC3000, *Psy* B728a and *Pph*1448A (Lindeberg et al., 2006). *Pto*DC3000 secretes around 28 T3SEs into plant hosts and the systematic deletion of them revealed extensive functional redundancy (Kvitko et al., 2009; Cunnac et al., 2011), which may be a result of the evolutionary history of the arms race between the pathogen and host. Even though the virulence mechanisms of T3SEs are extremely diverse, they seem to be converging on limited virulence pathways (Mukhtar et al., 2011; Wessling et al., 2014). In the following

sections, different T3SEs are discussed according to their virulence targets (Figure v), however it should be noted that a T3SE may evolve to target multiple immune regulators.

T3SEs targeting perception and signal transduction of defense response

AvrPto and AvrPtoB are the best studied T3SEs that target PRRs for virulence with multiple mechanisms. On one hand, AvrPto and AvrPtoB interact with different PRRs including FLS2, EFR and BAK1. Such an interaction either inhibit the kinase activity of these PRRs or the formation of ligand-induced complex (Goehre et al., 2008; Shan et al., 2008; Xiang et al., 2008). In addition, the kinase domain of FLS2 can be ubiquitinated by the E3 ligase domain found on the C-terminus of AvrPtoB, leading to FLS2 degradation in a proteasome-dependent manner (Goehre et al., 2008) AvrPtoB also ubiquitinates ERF and CERK1 in Arabidopsis, however only CERK was shown to be degraded as a result (Goehre et al., 2008; Gimenez-Ibanez et al., 2009).

Even though BAK1 is targeted by AvrPto, contrasting report suggests that FLS2, instead of BAK1, is the actual virulence target of AvrPto (Xiang et al., 2011). Whether BAK1 is a true virulence target of AvrPto is still debatable, BAK1 indeed interacts with multiple T3SEs. For instances, HopF2 is a mono-ADP-ribosyltransferase that suppresses PTI responses (Wu et al., 2011). HopF2 targets BAK1 and suppresses immunity by a still unknown mechanism (Zhou et al., 2014). Notably, the rice homolog OsBAK1 is also targeted by Xoo2875 (Yamaguchi et al., 2013a). As BAK1 is implicated in diverse roles from PTI, cell death and phytohormone signaling, BAK1 is indeed an attractive virulence target of T3SEs.

PRRs have long been shown to be phosphorylated on serine and threonine residues. However, researches on the roles of tyrosine phosphorylation in different PRRs do not gain its momentum until recently (Oh et al., 2009; Oh et al., 2010; Jaillais et al., 2011; Lin et al., 2014). By using a tyrosine kinase inhibitor tyrphostin A23 (A23) and phosphorylated tyrosine specific antibody, HopAO1was shown to be a tyrosine phosphatase that dephosphorylates a tyrosine residue on EFR to inhibit its function during activation (Macho et al., 2014).

Apart from the membrane localized PRRs, different receptor-like cytoplasmic kinases (RLKs) are also involved in innate immunity (Wu and Zhou, 2013). AvrPphB from *Pph*1448A is a cysteine protease that inhibits PTI by cleaving multiple RLKs including BIK1 and PBL1 for virulence. However, the cleavage of a RLK, PBS1, also triggers RPS5-dependent ETI response, again demonstrating the dual roles of T3SEs as both a virulence factor and an avirulence determinant in the presence of cognate R protein (Shao et al., 2003; Zhang et al., 2010). AvrAC is an uridylyl transferase that inhibits the kinase activity of PBL1 and RIPK by adding a uridine 5'-monophosphate to the phosphorylation sites in the activation loop of BIK1 and RIPK (Feng et al., 2012). Similar to AvrPphB, AvrAC also triggers ETI dependent on the RLKs PBL2 and RIPK (Guy et al., 2013). The T3E from *Xoo* MAFF311018 targets several rice RLCKs, including OsRLCK185. A downstream target of OsCERK1, OsRLCK18, is also targeted by the *Xanthomonas* T3SEs Xoo1488, but the underlying mechanism remains elusive (Yamaguchi et al., 2013b). RLKs correspond to a big gene family with only a few members are being demonstrated with an immune function; it is thus tempting to

speculate that the expansion of the RLK family is associated with increased surveillance capacity to detect different T3SEs that target RLKs for function. This hypothesis is supported by other examples including HopZ1a and ZED1. HopZ1a is an acetyltransferase (will be discussed in details in the section "YopJ family effectors and HopZ1a) that acetylates different proteins for virulence. However a pseudokinase ZED1 is evolved as a decoy to monitor HopZ1a activity, which in turns triggers ETI dependent on the CC-NB-LRR R protein ZAR1 (Lewis et al., 2010; Lewis et al., 2013).

MAPK signaling is one of the earliest responses activated upon flg22 perception (Meng and Zhang, 2013). Flg22 perception activates two MAPK cascades: one governed by MEKK1, MKK4/MKK5, and MPK3/MPK6 (Asai et al., 2002) and another one governed by MEKK1, MKK1/MKK2, and MPK4 (Ichimura et al., 2006; Suarez-Rodriguez et al., 2007). HopAI1 from *Pto*DC3000 irreversibly blocks MAPK signaling through its phosphothreonine lyase activity on MPK3, MPK4, MPK6 (Zhang et al., 2007). In addition to BAK1, HopF2 also targets MKK5 through ADP-ribosylation (Wang et al., 2010).

T3SEs targeting phytohormone pathways

As discussed in the previous section of bacterial toxins, phytohormone signaling plays important roles in fine-tuning defense against biotrophs and necrotrophs (Glazebrook, 2005). HopI1 is a J domain containing T3SE that suppresses the accumulation of SA in the chloroplast. J domain is found in co-chaperone including Hsp40 that interacts with and activates the ATPase activity of chaperon Hsp70 to regulate protein folding (Kelley, 1998). When expressed inside plants, HopI1 localizes to the

chloroplast and alters the thylakoid structure. HopI1 also affects the localization of Hsp70, which is required for HopI1 virulence function. However, how HopI1 inhibits the accumulation of SA is still unclear (Jelenska et al., 2007; Jelenska et al., 2010).

XopJ from *Xcv* is a protease that degrades RPT6, the 19S regulatory subunit of the proteasome. Consistent with the role of proteasome in regulating NPR1 turnover, XopJ triggers the accumulation of ubiquitinated NPR1 and suppresses SA dependent defense (Üstün et al., 2013; Ustun and Bornke, 2015).

HopZ1a from *P. syringae* pv. *syringae* A2 is an acetyltransferase that interacts with multiple JAZs through the Jas domain; the conserved C-terminal domain required for JAZ degradation. HopZ1a acetylates JAZs and lead to their degradation in a COI1-dependent manner. Expression of HopZ1a activates different JA responsive genes and suppresses the SA biosynthetic genes ICS1(Jiang et al., 2013). However, how acetylation can mediate JAZs degradation is unclear. Another T3SE, HopX1 also activates JA signaling via degradation of JAZs from its cysteine protease activity (Gimenez-Ibanez et al., 2014). Overall, these two effectors indirectly suppress the major defense signaling against biotrophs by derepressing JA signaling. Recently, AvrB was shown to promote the association between JAZ and COI1 and lead to the degradation of several JAZs. In addition, AvrB also reopens closed stomata through additional targeting on RIN4 and H^+-ATPase (Zhou et al., 2015b), which was previously shown to regulate stomata aperture (Liu et al., 2009b). This adds AvrB to the current list of T3SEs that target JAZs for virulence.

XopD from *Xcv* targets the tomato transcription factor SlERF4, which regulates ethylene production. SlERF4 is posttranslational modified by sumoylation. XopD destabilizes SlERF4 through its desumoylation activity. Silencing of SlERF4 reduces ET production and promotes susceptibility to *Xcv* and symptom development, suggesting that ET signaling positively regulating defense against *Xcv*. Therefore XopD actively suppresses ET-dependent defense by inhibiting ET production (Kim et al., 2013).

Apart from SA, JA and ET, other hormones signaling involving abscisic acid (ABA), auxin and cytokinin (CK) are also shown to be involved in defense response. Transgenic plants expressing HopAM1 exhibits ABA hypersensitive phenotype. Consistent with the role of ABA in regulating both biotic and abiotic stress response, HopAM1 promotes the virulence of *P. syringae* more under water-stressed condition (Goel et al., 2008). In general, auxin signaling negatively regulate defense against biotrophs. Flg22 perception induces the biogenesis of microRNA393 (miR393) which targets the auxin receptor TIR1 mRNA for cleavage. TIR1 is part of the ubiquitin ligase complex that destabilizes IAA, the negative regulator of auxin signaling. mi393-mediated TIR1 degradation suppresses auxin signaling and promotes resistance (Navarro et al., 2006). Several T3SEs are known to target auxin signaling independently. For examples, AvrPtoB suppresses miRNA393 transcription independent of its E3 ligase activity; AvrPto interferes with the maturation of miRNA by a still unknown mechanism and HopT1-1 inhibited miRNA mediated posttranslational inhibition (Navarro et al., 2008). Instead of targeting the miRNA pathway, AvrRpt2 promotes the accumulation of auxin (Chen et al., 2007) and enhances auxin signaling through stimulation of IAA turnover via

the 26S proteasome pathway (Cui et al., 2013). HopM1 targets ADP ribosylation factor (ARF) guanine nucleotide exchange factor AtMIN7 for degradation. As AtMIN7 was previously shown to recycle the auxin efflux carrier PIN1, HopM1 may promote plant susceptibility by disrupting polar auxin flux (Nomura et al., 2006).

HopQ1 homologs are widespread in different bacterial genera. HopQ1 mimics a CK biosynthetic enzyme to promote CK accumulation, which subsequently represses the expression of FLS2 (Hann et al., 2014). Therefore HopQ1 effectively dampens PTI response through manipulation of the CK pathway.

T3SEs targeting other defense related processes

HopM1 promotes the ubiquitination and degradation of AtMIN7. As AtMIN7 is an adenosine diphosphate ribosylation factor-guanine nucleotide exchange factor (ARF-GEF) that is involved in plant vesicle trafficking, HopM1-mediated AtMIN7 degradation disrupts the export of defense related compounds to the apoplast (Nomura et al., 2006; Nomura et al., 2011). In addition, HopM1 suppresses ROS burst and inhibits stomatal closure independent of AtMIN7, but the underlying mechanism is still unknown (Lozano-Duran et al., 2014). HopW1 is another T3SE that affects vesicle trafficking. HopW1 associates with actin and disrupts cytoskeleton during pathogen infection. As such, HopWI interferes with actin-dependent protein trafficking process. Such a phenotype is similar to plants treated with the inhibitor of actin polymerization, latrunculin B, indicating a possible suppression mechanism through direct targeting on actin (Jelenska et al., 2014). The acetyltransferase HopZ1a as discussed before also associates with tubulin and interferes with plant secretion pathway (Lee et al., 2012), AvrBsT acetylates

a microtubule associating protein and affects its localization (Cheong et al., 2014). These examples suggest that the cytoskeletal network and vesicle trafficking can be important factors in regulating defense.

Like HopF2, HopU1 is another ADP-ribosyltransferase that modifies glycine-rich RNA-binding protein 7 (GRP7) and inhibits its RNA binding activity (Fu et al., 2007). More importantly, HopU1 disrupts the association between GRP7 and FLS2, EFR mRNA, which is associated with their reduced protein levels. Since GRP7 also associates with different components involved in translation, HopU1 possibly affect the accumulation of FLS2 at the translation level (Nicaise et al., 2013)

AvrRpt2 is a cysteine protease that cleaves RIN4 into three fragments and triggers ETI in a RPS2 dependent manner (Axtell et al., 2003; Axtell and Staskawicz, 2003; Mackey et al., 2003; Chisholm et al., 2005). However, such an approach seems to be self-defeating as RIN4 is a negative regulator of defense as evidenced by the enhanced flg22-induced callose deposition in *rin4* mutant (Belkhadir et al., 2004; Kim et al., 2005). More recent study on the function of the cleaved products of RIN4 helps to reconcile this contradiction. RIN4 contains two nitrate inducing domains (NOI) at the N-terminus and C-terminus that are evolutionary distinct from each other. Upon cleavage by AvrRpt2, the NOI containing fragments have the ability to suppress PTI. However these two fragments seem to differ in their ability to suppress PTI and the removal of NOI domain does not render them unable to promote growth of nonvirulent strain *hrcC* or suppress callose deposition (Afzal et al., 2011). Targeting of RIN4 also imposes additional advantages. For instances, RIN4 also regulates the activity of proton ATPases AHA1 and AHA2,

which in turns regulate stomatal aperture (Liu et al., 2009a), making this multifunction protein an attractive target of several T3SEs.

HopN1 is a T3SE that is localized in the chloroplast. In tomato, HopN1 interacts with a component of photosystem II, PsbQ. HopN1 also inhibits the activity of photosystem II, possibly correlated with its PsbQ proteolysis activity *in vitro*. However no significant growth promotion activity can be attributed by HopN1 in *Pto*DC3000, but possible masking of virulence function by redundant effectors cannot be ruled out (Rodriguez-Herva et al., 2012).

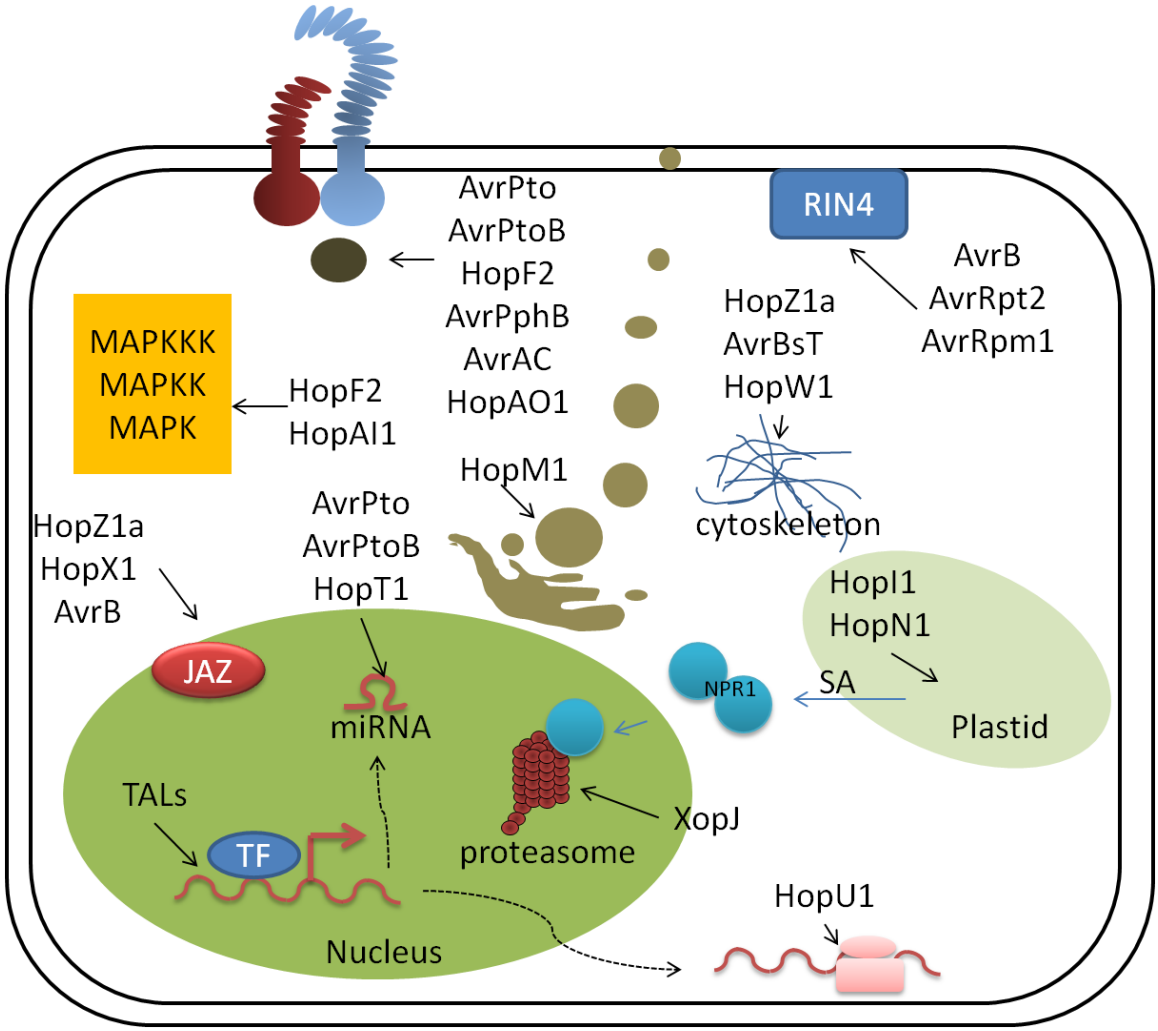
T3SEs as transcription activators

Transcription activator-like (TAL) effectors are T3SE from the genus *Xanthomonas*. Unlike other T3SEs discussed above, TAL effectors does not modify host target using enzymatic activities, instead they serve as transcription factors to activate genes that promote susceptibility (Doyle et al., 2013). TAL effectors are characterized by three parts: the central tandem repeats region, nuclear localization signal and acidic activation domain at the C-terminus. Each tandem repeat unit consists of 33-34 amino acids with two variable amino acids found at position 12 and 13 often known as repeat variable diresidue (RVD). Each RVD in turns corresponds to a single nucleotide inside the nucleus (Boch et al., 2009; Moscou and Bogdanove, 2009). The crystal structure of the natural TAL effector PthXo1 reveals that each repeat unit forms a two-helix bundle with the RVD located at the centre of the helical loop. The two-helix then wraps around an unbent DNA with direct contact between the nucleotide and the RVD (Deng et al., 2012; Mak et al., 2012).

Targets of TAL effectors include a master regulator of cell size, UPA20 and SWEET proteins. Activation of *UP20* induces hypertrophy that may promote bacterial dispersal (Wichmann and Bergelson, 2004; Kay et al., 2007); whereas SWEET may potentially enhance availability of sugar to bacteria (Verdier et al., 2012).

Figure v. Manipulation of different components of defense by T3SEs.

A conceptual illustration of different virulence targets of T3SEs. Refer to the main text for more explanations.



YopJ family effectors are acetyltransferases

YopJ is one of the largest T3SE family with members widespread in both animal and plant pathogens (Ma et al., 2006). The archetypal member YopJ from the animal pathogen *Yersinia pestis* is notorious as the causative agent of black plague claiming millions of life back in the 13th century. As a potent virulence factor, YopJ inhibits animal innate immunity through direct targeting of several MAPK. YopJ acetylates several residues that coincide with the phosphorylation sites, leading to the blockage of MAKP signaling event (Mukherjee et al., 2006). Several other YopJ family effectors including AvrA from *Salmonella typhimurium,* VopA from *Vibrio parahemeolyticus,* AvrBsT from *Xcv,* HopZ1a from *Psy*A2 and PopP2 from *R. solanacearum* also uses acetyltransferase activities to target different proteins for virulence (Trosky et al., 2007; Jones et al., 2008; Lee et al., 2012; Jiang et al., 2013; Cheong et al., 2014; Le Roux et al., 2015; Sarris et al., 2015).

YopJ family effectors contain a conserved catalytic triad consisting of histidine (His), glutamate (Glu) and cysteine (Cys) and mutation of the cysteine residues completely abolish the biological function of this group of effector (Orth et al., 2000; Ma et al., 2006; Jones et al., 2008; Cheong et al., 2014). Sequence alignment of YopJ effectors reveals a relatively conserved central catalytic domain and a C-terminal region with undefined function (Figure vi). Given that the high similarity between the predicted secondary structures of YopJ that of adenovirus protease (AVP), YopJ effectors were initially proposed to be cysteine protease (Orth et al., 2000). Indeed, weak protease activities are detected by different YopJ effectors *in vitro* (Ma et al., 2006). Provided that

both acetyltransferase and cysteine protease activities both require a conserved catalytic triad for enzymatic activity, it is not surprising to find another YopJ family effector, XopJ, acting as a cysteine protease for virulence (Ustun and Bornke, 2015). Interestingly, the *in vitro* cysteine protease activity can be enhanced by the addition of plant extract, suggesting that a potential host factor may promote YopJ effector activity (Ma et al., 2006). Using a combination of chromatography and mass spectrometry techniques, this host factor is later found to be inositol hexakisphosphate (IP6). Addition of IP6 induces conformational change of AvrA and activation of enzymatic activities of both AvrA and YopJ (Mittal et al., 2010).

The two-step "ping-pong" model was originally proposed to account for the acetyltransferase activity of YopJ (Mukherjee et al., 2007). This model depicts the formation of an acetyl-enzyme intermediate followed by the transfer of the acetyl group to the substrate. This model seems to generally apply to other YopJ family effectors as acetyl-enzyme intermediates are found among all YopJ effectors with demonstrated acetyltransferase activity (Mittal et al., 2010; Tasset et al., 2010; Lee et al., 2012; Cheong et al., 2014). A conserved lysine residue in PopP2, K383, is auto-acetylated and required for PopP2 autoacetylation (Tasset et al., 2010). Mutation of the corresponding lysine in other YopJ effector, K289 of HopZ1a and K282 of AvrBsT, also affect their biological function, however whether these residues serve as actual auto-acetylation sites is not demonstrated. In Chapter I, I will demonstrate how various biochemical, genetic and plant pathology approaches were employed to identify and characterize the auto-

acetylation site of HopZ1a and study two novel residues required for HopZ1a activation and interaction with cofactor.

HopZ1a and its functions in plant hosts

HopZ1a is one of the best studied YopJ family effector found in phytopathogen. There are three alleles of HopZ1, HopZ1a, HopZ1b and HopZ1c (Ma et al., 2006). Phylogenetic study suggests that HopZ1a resembles the ancestral form of HopZ1 and strong selective forces exerted by the evolution of the cognate R protein drives the diversification of HopZ1. Even though HopZ1a and HopZ1b share approximately 81% similarity in their amino acid sequence, they differ in their enzymatic activity, virulence targets and HR-elicitation properties. More recently, different HopZ effectors including HopZ1c, HopZ2 and HopZ3 were shown to suppress PTI, but the underlying mechanism and virulence targets are unclear (Lewis et al., 2014).

In *Arabidopsis* eco. Col-0 and soybean cultivar Williams 82, only HopZ1a but not HopZ1b triggers HR. In *N. benthamiana*, both HopZ1a and HopZ1b triggers cell death. However, only HopZ1b-triggered cell death can be suppressed by another YopJ family effector, HopZ3, indicating that HopZ1a- and HopZ1b-mediated defense are activated by different pathways (Zhou et al., 2009). HopZ1a-mediated HR in Arabidopsis is attributed by the R protein ZAR1 and no close homolog of ZAR1 can be found in soybean (Lewis et al., 2010; Lewis et al., 2013). This further supports the idea that HopZ1a and HopZ1b are differentially recognized in different systems. Chimeric study exchanging different domains of HopZ1a and HopZ1b also reveals the requirement of the central region spanning across the catalytic triad as avirulence determinant (Morgan et al., 2010). The

N-terminal glycine residue, G2, is required for myristolation, membrane localization and elicitation of HR in Arabidopsis (Lewis et al., 2008). However the HopZ1a(G2A) mutant is still able to promote bacterial growth, suggesting that membrane localization of HopZ1a can be uncoupled from its virulence function (Jiang et al., 2013).

In terms of their virulence function, HopZ1a is a much stronger acetyltransferase than HopZ1b (refer to "Results" of Chapter I). In Arabidopsis, HopZ1a acetylates JAZs and promotes virulence through the SA pathway. Alternatively, HopZ1a also acetylates tubulin and disrupts microtubulin network for virulence (Lee et al., 2012; Jiang et al., 2013). In soybean, both HopZ1a and HopZ1b target an isoflavone biosynthetic gene, GmHID1, and trigger its degradation without any acetyltransferase activity demonstrated. GmHID1 degradation is also associated with a decrease of daidzein production (Zhou et al., 2011). Since daidzein is a precursor of antimicrobial phytoalexin (Akashi et al., 2005; Lozovaya et al., 2007; Bednarek and Osbourn, 2009), suppression of isoflavone biosynthesis dampens secondary metabolites-mediated defense. In order to understand more about virulence function of HopZ1a and HopZ1b in soybean, yeast-two-hybrid was carried out by my colleague Dr. Huanbin Zhou and GmZINP2, a protein with potential chromatin remodeling function, was identified.

Chromatin as another battlefield for pathogen-host interaction

Eukaryotic chromatin is a very dynamic structure and modifications on histone and DNA both contribute to global transcription reprogramming (Ma et al., 2011). In eukaryotes, the genetic information stored on DNA is associated with various proteins. For instance, DNA is wrapped around an octamer of core histone proteins composed of

H2A, H2B, H3 and H4 in the form of "bead-on-a-string" model. The linker protein H1 stabilizes this interaction to form the single unit coined nucleasome. Nucleasomes are further condensed in the form of fiber. Collectively, different hierarchs of highly condensed nucleoprotein complexes form the dynamic eukaryotic chromatin. The accessibility of DNA to different transcription regulators can be regulated in at least three levels (Figure vii) (Margueron and Reinberg, 2010).1) DNA can be directly methylated and such a modification is often associated with gene silencing process (Schubeler, 2015); 2) The tails of core histone are subjected to modification through acetylation and methylation. In general, histone acetylation enhances transcription, possibly by attenuation of interaction between histone proteins and DNA; whereas the roles of methylation on histones are highly variable. In addition, histone can be modified in forms of phosphorylation, sumoylation, ubiquitination, glycosylation and ADP-ribosylation. The different modification marks on histone contribute to unique histone map that marks a region for transcription reprogramming (Fuchs et al., 2006; Berger, 2007; Bannister and Kouzarides, 2011; Hottiger, 2011); 3) A group of ATP driven chromatin remodeling protein can directly alter the configuration of chromatin by various mechanisms (Jerzmanowski, 2007; Clapier and Cairns, 2009; Hargreaves and Crabtree, 2011; Gentry and Hennig, 2014). Together these mechanisms can fine tune the transcription level of a specific locus or global transcriptome.

In plants, several ATP-dependent chromatin-remodeling proteins are implicated in the regulation of plant innate immunity (Figure viii). SYD and BRM both regulate expression of defense related genes. Among them, *PR1* is up-regulated in the *syd-2* and

brm-101 mutants, suggesting that they may negatively regulate defense response (Bezhani et al., 2007; Walley et al., 2008). It is likely that SYD is recruited to the promoters of these genes, affecting their transcription through its chromatin remodeling activity (Walley et al., 2008). In the contrary, the effect of BRM on *PR1* expression is likely indirect. A bromodomain and several DNA binding regions are found in BRM, which may govern BRM binding specificity to different loci (Farrona et al., 2007). BRM has been shown to activate gibberellins (GA) signaling through binding to promoters of specific positive regulators (Archacki et al., 2013). Another chromatin-remodeling proteins, SWI3C, brings the GA suppressor DELLA and its modifier SPY together, suggesting that SWI3C may regulate DELLA function through the recruitment of additional regulator(s) (Sarnowska et al., 2013).

PIE1 is a negative regulator of defense against *Pto*DC3000 that has been shown to deposit the histone variant H2A.Z to PIE1-regulated loci. It is possible that PIE1 deposits H2A.Z at defense-related loci and suppresses their transcription in the absence of pathogen infection (Deal et al., 2007; March-Diaz et al., 2008). Notably, another component in the PIE associated-chromatin remodeling complex, ARP6, is involved to regulate defense response in a temperature-dependent manner (Cheng et al., 2013). However, the mechanism underlying the dynamics of exchange between H2A and H2A.Z during defense response is unclear. DDM1 is another chromatin remodeling protein required to maintain DNA methylation, possibly through modulation of DNA accessibility to methyltransferases and demethylases (Brzeski and Jerzmanowski, 2003; Zemach et al., 2005). DDM1 and SYD also regulate the expression of the receptor SNC1,

which induces defense response upon activation (Li et al., 2010; Johnson et al., 2015). However the underlying mechanism on how DDM1 and SYD regulate SNC1 expression remains elusive.

Our knowledge on how bacteria may target chromatin related components for virulence is very limited. Apart from the TAL effectors that serve as transcription activators as discussed in the previous section, so far no T3SE is shown to directly modulate chromatin for virulence. In Chapter II, I will demonstrate how HopZ1a may potentially affect GmZINP2 function.

Figure vi. Multiple sequence alignments of YopJ effectors.

Alignment of YopJ family effectors. Sequences were aligned using Clustal omega with conserved residues highlighted as blue.

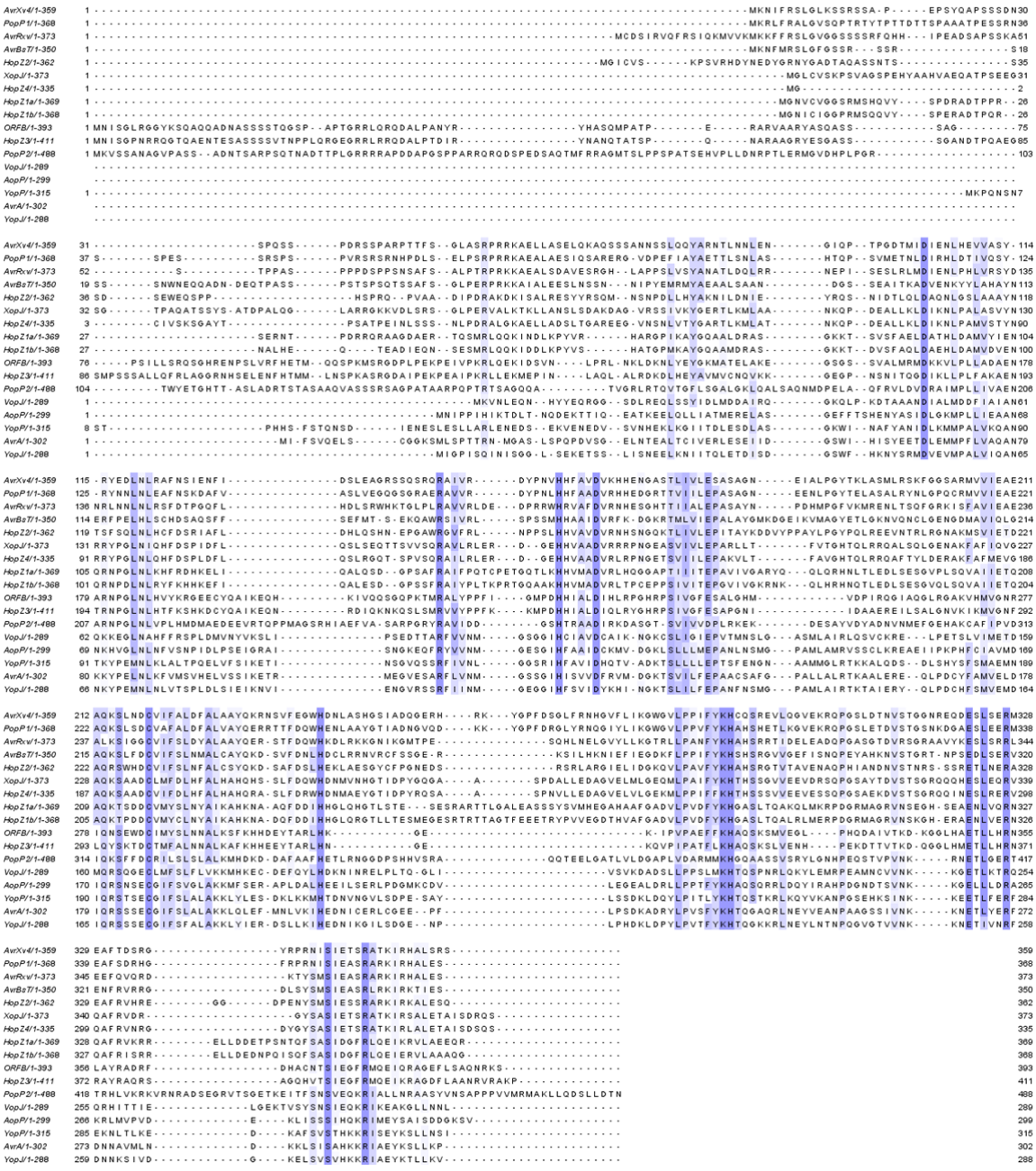


Figure vii. Schematic representation of chromatin and epigenetic regulation. Eukaryotic chromain are under different levels of regulation. Transcription can be repressed by methylation on DNA. Tails of histone are subjected to various post-translational modification (PTMs), leading to alterations in chromatin structures. The DNA accessibility can also be regulated by chromatin remodeling protein through mechanism from sliding, replacement of histone variants and displacement.

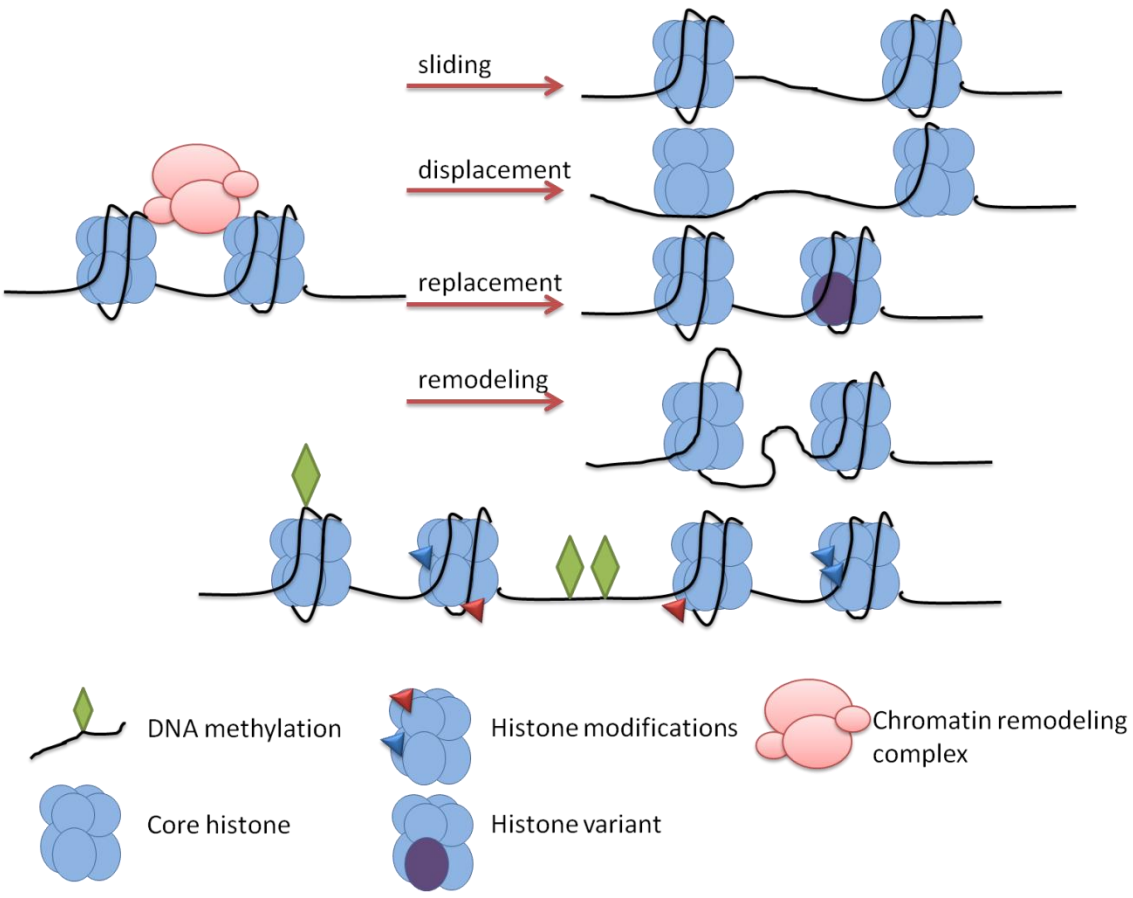


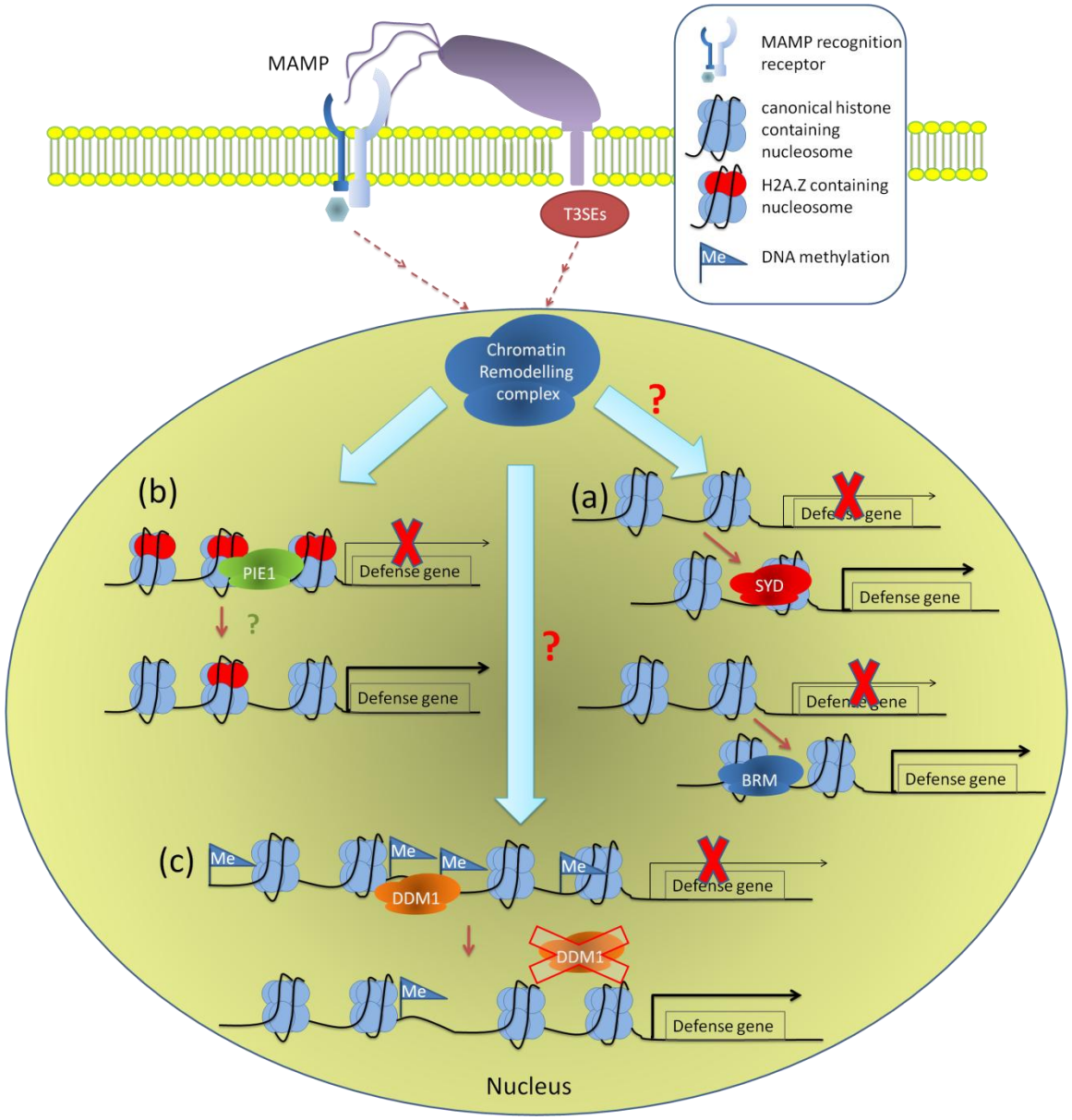
Figure viii. Chromatin remodeling proteins involved in the regulation of plant defense.

(Adopted from Ma *et al*., *Plant Physiology*. 2011). Possible mechanisms of ATP dependent chromatin remodelers in regulating plant immunity upon bacterial infection. Chromatin remodelers are activated or repressed in response to pathogen perception by unknown pathways.

A, SYD, and possibly BRM, are recruited to target loci and induces the expression of defense-related genes.

B, PIE1 mediates H2A.Z deposition at target loci in the absence of pathogens to suppress defense gene expression. Upon pathogen infection, these genes might be activated due to a decrease of H2A.Z occupancy level.

C, DNA methylation-associated gene silencing on defense genes might be alleviated due to the down-regulation of DDM1 at the transcriptional level upon pathogen infection.


MAMP
T3SEs
MAMP recognition receptor
canonical histone containing nucleosome
H2A.Z containing nucleosome
Me
DNA methylation
Chromatin Remodelling complex
?
(b)
PIE1
Defense gene
?
Defense gene
?
(a)
Defense gene
SYD
Defense gene
Defense gene
BRM
Defense gene
(c)
Me
Me
Me
Me
DDM1
Defense gene
DDM1
Me
Defense gene
Nucleus

.

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Chapter I. Two serine residues in *Pseudomonas syringae* effector HopZ1a are required for acetyltransferase activity and association with the host co-factor

(Ma KW, Jiang S, Hawara E, Lee D, Pan S, Coaker G, Song J, Ma W. 2015. *New Phytologist*. DOI: 10.1111/nph.13528)

ABSTRACT

Gram-negative bacteria inject type III secreted effectors (T3SEs) into host cells to manipulate the host immune response. The YopJ family effector HopZ1a produced by the phytopathogen *Pseudomonas syringae* possesses acetyltransferase activity and acetylates plant proteins to facilitate infection. All YopJ effectors with acetyltransferase activities require a conserved catalytic triad of histidine, glutamate and cysteine for function. However, comprehensive study on residues/domain required for HopZ1a acetyltransferase activity is lacking. Using mass spectrometry, I ruled out the possibility of a previously identified residue, K289 as an autoacetylation site of HopZ1a. Instead, I identified a threonine residue, T346, as the main autoacetylation site of HopZ1a. Two neighboring serine residues, S349 and S351, are required for the acetyltransferase activity of HopZ1a *in vitro* and are indispensable for the virulence function of HopZ1a in *Arabidopsis thaliana*. Using proton nuclear magnetic resonance (NMR), I observed a conformational change of HopZ1a in the presence of inositol hexakisphosphate (IP6), which acts as a eukaryotic co-factor and significantly enhances the acetyltransferase

activity of several YopJ family effectors. S349 and S351 are required for IP6-binding-mediated conformational change of HopZ1a. S349 and S351 are located in a conserved region in the C-terminal domain of YopJ family effectors. Mutations of the corresponding serine(s) in two other effectors, HopZ3 of *P. syringae* and PopP2 of *Ralstonia solanacearum*, also abolished their acetyltransferase activity. My results highlight the significances of additional residues other than the conserved catalytic triad required for YopJ family effector function.

INTRODUCTION

Gram negative bacteria use the T3SS to secrete different T3SEs into eukaryotic host to promote virulence. The YopJ family of T3SEs has been identified from a large variety of animal and plant pathogens, including *Vibrio*, *Yersinia*, *Aeromonas*, *Salmonella*, *Pseudomonas*, *Ralstonia*, *Xanthomonas* and *Pectobacterium* (Ma et al., 2006). YopJ family effectors share a conserved catalytic triad consisting of histidine, glutamate and cysteine. The catalytic cysteine residue is unexceptionally required for the enzymatic activity and biological function of YopJ family effectors. Although the catalytic triad of YopJ family effectors is identical to that of the C55 family of cysteine proteases (Orth et al., 2000), several family members have been shown to act as acetyltransferase and modify target proteins in their hosts through acetylation (Mittal et al., 2006; Mukherjee et al., 2006; Trosky et al., 2007; Jones et al., 2008; Lee et al., 2012; Jiang et al., 2013; Lewis et al., 2013; Cheong et al., 2014).

YopJ, the founding member of this effector family from *Yersinia pestis*, acetylates specific lysine, serine and threonine residues of several mitogen activated protein kinases in animals (Mittal et al., 2006; Mukherjee et al., 2006). Some of the serine and threonine acetylation sites coincide with phosphorylation sites within the activation loop of the corresponding kinases. As such, YopJ suppresses immune signaling by blocking the mitogen activated kinase phosphorylation relay. In addition to YopJ, AvrA from *Salmonella typhimurium* and VopA from *Vibrio parahemeolyticus* also acetylate target kinases and suppress immune signaling in mammalian cells (Trosky et al., 2007; Jones et

al., 2008). YopJ family effectors produced by plant pathogens are also able to modify their host targets. For example, AvrBsT from *Xanthomonas euvesicatoria* acetylates the microtubule associated protein ACIP in Arabidopsis and affects its subcellular localization (Cheong et al., 2014).

HopZ1a is a YopJ-like effector produced by the plant pathogen *Pseudomonas syringae*. HopZ1a possesses a weak cysteine protease activity using a generic substrate *in vitro* (Ma et al., 2006); however, it also acts as an acetyltransferase and modifies several plant substrates including tubulin (Lee et al., 2012), Jasmonate ZIM (JAZ) domain proteins (Jiang et al., 2013), and the pseudokinase ZED1 (Lewis et al., 2013).

YopJ family acetyltransferases use acetyl-coenzyme A (acetyl-CoA) as the acetyl group donor. In general, two mechanisms have been proposed to account for the acetyl-CoA-dependent acetyltransferase activity (Berndsen and Denu, 2005). The first one involves the formation of a ternary complex with a lysine on the enzyme attacking the bound acetyl-CoA and subsequently transferring the acetyl group to the substrate. The second mechanism is called the "ping-pong" model, which involves formation of an acetyl-enzyme intermediate on the catalytic cysteine residue; the acetyl group is then transferred to a substrate. This latter mechanism has been proposed to be used by YopJ (Mukherjee et al., 2006). In support of this hypothesis, autoacetylation was observed in all the YopJ family effectors with demonstrated acetyltransferase activity (Mittal et al., 2010; Tasset et al., 2010; Lee et al., 2012; Cheong et al., 2014). If this model is correct, the autoacetylation sites, at least some of them, would also be required for *trans* acetylation.

The autoacetylation site of a YopJ family effector, PopP2, has been determined by mass spectrometry as lysine383 following the catalytic triad (Tasset et al., 2010). This finding suggests that residues other than the catalytic cysteine can potentially form the acetyl-enzyme intermediate and subsequently transfer the acetyl group to a substrate. K383 is conserved among most YopJ effectors, including HopZ1a. Substitution of the corresponding residue lysine289 in HopZ1a with an arginine leads to loss of HR-triggering and bacterial growth promoting activities in Arabidopsis (Lee et al., 2012), suggesting that the conserved lysine is also required for the biological function of HopZ1a. However, whether K289 is the autoacetylation site in HopZ1a remains unclear.

In this study, I characterized key residues that contribute to the acetyltransferase activity, co-factor interaction, and biological functions of HopZ1a. The results showed that K289 is not an autoacetylation site in HopZ1a. Using mass spectrometry, I identified a threonine residue, T346, in the C-terminal region as the main autoacetylation site. In addition, I found that two neighboring serine residues, S349 and S351, play a key role in the enzymatic activity and virulence function of HopZ1a. Importantly, these two serine residues are essential for the interaction of HopZ1a with the co-factor inositol hexakisphosphate (IP6), which has been shown to activate the acetyltransferase activity of several YopJ family effectors in eukaryotic hosts (Mittal et al., 2010; Lee et al., 2012; Cheong et al., 2014). Finally, I demonstrate that S349 and S351 are highly conserved among members of the YopJ family and are also required for the acetyltransferase activity of HopZ3 produced by *P. syringae* pv. *syringae* and PopP2 produced by *Ralstonia solanacearum*. These results suggest that, in addition to the highly conserved

catalytic residues, YopJ family effectors also require conserved serine(s) in the C-terminal domain for their enzymatic activity.

MATERIALS AND METHODS

Bacterial strains and plasmids

Pseudomonas syringae pv .*tomato* DC3000 (*Pto*DC3000), *P. syringae* pv .*tomato*D28E (*Pto*D28E), *P. syringae* pv .*syringae B728AΔZ3* (*Psy*B728AΔZ3), *P. syringae* pv .*glycinea* BR1O1 (*Pgy*BR1O1), were grown at 30℃ in King's B medium (King et al, 1954) (King et al., 1954) or M63 minimal medium containing 1% fructose for the induction of T3SS as reported (Morgan et al., 2010). *Agrobacterium tumefaciens* (C58C1) and *Escherichia coli* strains DH5α and BL21 were grown in Luria–Bertani (LB) medium at30℃ and 37℃ as described (Morgan et al., 2010). *P. syringae* transformation was transformed by electroporation using an Eppendorf electroporator 2510 (Eppendorf North America, Westbury, NY, USA) according to the manufacturer's instructions. Bacteria strains and constructs used in this study are summarized in Table 1.1.

Plant materials and growth conditions

Arabidopsis thaliana (L.) Heynh. seeds were sown in soil and stratified at 4 ℃ for 3 days. The plants were grown in a conditioned growth room (19-21 ℃, 16-hour photoperiod, and relative humidity of 75-80%). Wild-type and mutant *HopZ1a* genes, tagged with 3xFLAG on the N terminus, were cloned into the vector pEG100 (Jiang et al., 2013). The recombinant plasmids were transformed into *Agrobacterium tumefaciens*. Arabidopsis *zar1-1* plants were transformed using the floral dip method (Clough and Bent, 1998).

Soybean (*Glycine max* (L.) cv. Williams 82) seeds were surface sterilized with 10% bleach for 10 min and pre-germinated on wet filter paper at room temperature in the dark for 4 days. Seedlings were transplanted to soil and grown in a conditioned growth room (19-21 ℃, 16-hour photoperiod, and relative humidity of 75-80%).

N. benthamiana seeds were germinated in soil. Seedlings were then transferred to big pots and gown in conditioned growth room (19-21 ℃, 16-hour photoperiod, and relative humidity of 75-80%)

Protein expression and purification

Wild-type and mutant *HopZ1a* and *PopP2* were cloned into the plasmid vector pRSFDuet-1 (Novagen, Madison, WI) containing a 6×His-SUMO tag, and transformed in *E. coli* strain BL21(DE3). Unless otherwise specified, all proteins were induced at OD_{600} = 0.6 (approximately 6×10^8 cfu/mL) with a final concentration of 1mM IPTG at 22°C overnight. Recombinant 6×His-SUMO-tagged proteins were purified using nickel resin, and the 6×His-SUMO tag was subsequently removed by ULP1 protease as described previously (Jiang et al., 2013).

For NMR samples, tag-free HopZ1a, HopZ1a(C216A), HopZ1a(K289R) and HopZ1a(S349AS351A) were further purified by size exclusion chromatography on a Superdex 200 HR 16/60 column (GE Health, Little Chalfont, UK), using buffer containing 20 mM sodium phosphate (pH 7.5) and 150 mM NaCl.

Wild-type and mutant *HopZ3* were cloned in pET14b vector and expressed in *E. coli* as a 6×His-tagged proteins. The 6×His-tagged HopZ3 proteins were purified using a nickel affinity column.

In vitro acetylation assays

An *in vitro* acetylation assay was used to examine the acetyltransferase activity of HopZ1a, HopZ1b, PopP2 and HopZ3 to determine the autoacetylation level. 1 μg HopZ1a, PopP2, 1.5 μg HopZ3 or 3ug of HopZ1b was incubated with 1 μL [14C]-acetyl-CoA (55 μci/μmol) in 25 μL of reaction buffer (50 mM HEPES (pH 8.0), 10% glycerol, 1 mM DTT, 1 mM PMSF, 10 mM sodium butyrate) at 30 ℃ for one hour. 100 nM IP6 was supplemented to the reaction when appropriate. To determine the *trans* acetylation, 7 μg MBP-AtJAZ6-HIS was used in each reaction as the substrate. The reactions were stopped by addition of 2x Laemmli buffer and then subjected to SDS-PAGE. Acetylated proteins were detected by autoradiography as previously described (Jiang et al., 2013). After autoradiography, the protein gels were removed from the filter paper and stained with Coomassie Blue as a loading control.

Nano-UPLC-MS/MS analysis

To identify the autoacetylation site(s) in HopZ1a, 1 μg of tag-free HopZ1a or HopZ1a(C216A) was incubated with C12 acetyl-CoA in a 25 μL reaction system for one hour. The proteins were precipitated in 100 μL of cold acetone overnight at -20 ℃. Protein pellets were collected with 30 minutes of centrifugation at18000g, washed with cold acetone, air-dried, and finally digested with 1 μg trypsin in 100 μL ammonium bicarbonate (50 mM, pH 8.0) at 37 ℃ overnight. The trypsin-digested samples were dried to pellets with a speedvac concentrator and then resuspended in 20 μL 0.1% formic acid solution. A MudPIT LC/MS method was employed to analyze these final peptide samples using two-dimension nanoAcquity UPLC (Waters, Milford, MA) and Orbitrap Fusion

mass spectrometer (Thermo Fishers, Waltham, MA). The two-dimension nano-UPLC fractionation and separation gradient was described in (Drakakaki et al., 2012) and the MS survey scan using data-dependent acquisition (DDA) was described previously (Hebert et al., 2014). To confirm the specific acetylation site, the same samples were re-analyzed using a targeted scan method, with which the MS2 spectra were acquired using Orbitrap, instead of ion trap, with a resolution set at 30,000.

All raw MS data were processed with Proteome Discoverer version 1.4 (Thermo Fishers, San Jose, CA) to generate mgf files that were used in a Mascot search against the HopZ1a protein sequence. Mascot search parameters allowed various modifications including N-terminal acetylation, oxidation (M), Gln->pyro-Glu (N-term Q), Glu->pyro-Glu (N-term E), K-acetylation, S-acetylation, T-acetylation and H-acetylation. HopZ1a(C216A) was used as a control to identify HopZ1a specific acetylation site(s).

***Pseudomonas syringae* infection assays**

The leaves of five-week old Arabidopsis plants were infiltrated with bacterial suspensions at OD_{600} = 0.0001 (approximately 1×10^5 cfu/mL) for *Pto*DC3000 and OD_{600} = 0.005 for *Psy*B728aΔ*Z3* (approximately 5×10^6 cfu/mL). The inoculated plants were transferred to a growth chamber (22 ℃ and 16/8 light/dark regime, 90% humidity), and the bacterial populations were determined as colony forming units (cfu) per cm^2 three days after inoculation using a previously described procedure (Morgan et al., 2010).

To test the HR-triggering activity of HopZ1a mutants, leaves of five-week old Arabidopsis plants (eco. Col-0), fully expanded primary leaves of 14-day old soybean (cultivar Williams 82) and fully expanded leaves of *N. benthamiana* were infiltrated with

bacterial suspensions at an OD_{600} = 0.2 (approximately 2×10^8 cfu/mL) for *Pto*D28E (Cunnac et al., 2011), OD_{600} = 0.1 (approximately 1×10^8 cfu/mL) for *Pgy*BR1-O1, OD_{600} = 0.05 (approximately 5×10^7 cfu/mL) together with the silencing suppressor P19 (Voinnet et al., 1999) for *A. tumefaciens* C58C1. Cell death symptoms within the infiltrated areas were monitored at 24 hpi for *Pto*D28E, 36 hpi for *Pgy*BR1-O1 and 24hpi for *A. tumefaciens* C58C1 hours after inoculation. Soybeans were cleared with 95% ethanol before the observation of cell death in soybean.

Callose deposition analysis

Leaves of five-week old transgenic Arabidopsis *zar1-1* plants expressing wild-type or mutant HopZ1a were infiltrated with water or 1μM flg22 (PhytoTechnology Laboratories, Shawnee Mission, KS). Sixteen hours after the treatment, the infiltrated leaves were fixed in an ethanol:acetic acid solution as previous described (Millet et al., 2010), and stained with 0.01% aniline blue (Sigma-Aldrich, St. Louis, MO). Callose deposition in the leaves was observed under UV using a BX51 fluorescent microscope (Olympus, Center Valley, PA). In each treatment, twelve independent images were analyzed and the numbers of callose deposits were numerated using ImageJ.

Stomata aperture analysis

Stomata apertures were analyzed using a modified protocol based on a procedure described previously (Liu et al., 2009). Leaf discs of four-week old transgenic Arabidopsis *zar1-1* plants expressing wild-type or mutant HopZ1a were incubated, with the abaxial side facing down, in a MES buffer (10 mM KCl, 0.2 mM $CaCl_2$, 10 mM MES-KOH (pH 6.5), 0.025% silvet-77). Full opening of the stomata was induced by

placing the discs under illumination for at least two hours before the buffer was replaced with fresh MES buffer containing 10 μM of flg22. Leaf discs were then incubated with flg22 under illumination for another two hours. Medical adhesive (Hollister, Libertyville IL) was applied on a slide and the leaf discs were placed on the adhesive with the abaxial side facing down. A razor blade was then used to carefully scrape away the upper epidermis and the stomata were immediately observed using a Primo Star microscope (Zeiss, Oberkochen, Germany). At least ten independent images were taken from each treatment and at least 6 stomata per image were analyzed for aperture, which was expressed as the ratio of width over length.

1D proton (^{1}H) NMR

For NMR experiments, 0.1 mM purified wild type and mutant HopZ1a proteins, in the absence or presence of 1 mM IP6, were dissolved in 500 μL buffer containing 20 mM sodium phosphate (pH 7.5), 150 mM NaCl and 10% D_2O. 1D proton NMR spectra, 256 scans each, were collected for HopZ1a proteins on a Bruker Advance 600 MHz NMR spectrometer equipped with a TXI probe at 25 ℃. The NMR spectra were then processed and analyzed using the Topspin software (Bruker Inc., Billerica, MA)

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

Strains or Plasmids	Description	Source/ reference
Escherichia coli DH5α	F- Φ80d*lac*ZΔM15 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. *tomato* strain DC3000	Isolated from tomato plants, also infects *Arabidopsis*, Rif^R	Cuppels, 1986
Pseudomonas syringae pv. *tomato* strain D28E	A mutant of *Pto*DC3000 that does not produce coronatine, Rif^R, Kan^R, $Spect^R$	Cunnac *et al.*, 2011
Pseudomonas syringae pv. *glycinea* BR1Rif-O1	A mutant of *Pgy*BR1, Rifampicin spontaneous mutant with the endogenous plasmid carrying HopZ1b cured	Zhou *et al* ., 2009
Pseudomonas syringae pv. *syringae* B728aΔZ3	A mutant *Psy*B728a unmarked deletion of HopZ3, Rif^R	Vinatzer *et al* .2006
Agrobacterium tumefaciens GV3101(pMP90)	Rif^R, $Gent^R$	Holsters, 1980
Agrobacterium tumefaciens C58C1 (pCH32)	Rif^R, Tet^R	Mudgett *et al.*, 2000
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::*HopZ1a(K289R)-HA*	pUCP20tk carrying the gene encoding *HopZ1a* tagged with HA with lys289 replaced with an arginine, Kan^R	this study
pUCP20tk::*HopZ1a(S349AS351A)-HA*	pUCP20tk carrying the gene encoding *HopZ1a* tagged with HA with Ser349 and Ser351 replaced with alanine, Kan^R	Shushu Jiang

pUCP20tk::*HopZ1a(S344A)-HA*	pUCP20tk carrying the gene encoding *HopZ1a* tagged with HA with Ser344 replaced with alanine, Kan^R	this study
pUCP20tk::*HopZ1a(T346A)-HA*	pUCP20tk carrying the gene encoding *HopZ1a* tagged with HA with Thr346 replaced with alanine, Kan^R	this study
pUCP20tk::*HopZ1a(S344AT346A)-HA*	pUCP20tk carrying the gene encoding *HopZ1a* tagged with HA with Ser344 and Thr346 replaced with alanine, Kan^R	this study
pUCP20tk::*HopZ1a(S349A)-HA*	pUCP20tk carrying the gene encoding *HopZ1a* tagged with HA with Ser349 replaced with alanine, Kan^R	this study
pUCP20tk::*HopZ1a(S351A)-HA*	pUCP20tk carrying the gene encoding *HopZ1a* tagged with HA with Ser351 replaced with alanine, Kan^R	this study
pUCP20tk::*HopZ1a(K289RS349AS351A)-HA*	pUCP20tk carrying the gene encoding *HopZ1a* tagged with HA with Lys289 replaced with an arginine and Ser349 and Ser351 replaced with alanine, Kan^R	Shushu Jiang
pEG100::*HopZ1a-HA*	pEG100 carrying *hopZ1a* tagged with HA at C-terminus, Kan^R	Jiang *et al.*, 2013
pEG100::*HopZ1a(C216A)-HA*	pEG100 carrying *hopZ1a(C216A)* tagged with HA at C-terminus, Kan^R	Jiang *et al.*, 2013
pEG100::*HopZ1a(K289R)-HA*	pEG100 carrying *hopZ1a(K289R)* tagged with HA at C-terminus, Kan^R	this study
pEG100::*HopZ1a(S349AS351A)-HA*	pEG100 carrying *hopZ1a(S349AS351A)* tagged HA at C-terminus, Kan^R	this study
pEG100::*HopZ1a(K289RS349AS351A)-HA*	pEG100 carrying *hopZ1a(K289RS349AS351A)* tagged with HA at C-terminus, Kan^R	this study
pGEX4T-2::*HopZ1a*	pGEX4T-2 carrying *hopZ1a*, Amp^R	Zhou *et al.*, 2011
pET-mal::*AtJAZ6*	pET-mal carrying *AtJAZ* genes, Kan^R	Jiang *et al.*, 2013
pRSF::*HopZ1a(32-326)*	N-terminal 6×His-SUMO tagged *HopZ1a* with deletion of N-terminal 31 aa. Kan^R	Jiang *et al.*, 2013
pRSF::*HopZ1a(C216A)(32-326)*	N-terminal 6×His-SUMO tagged *HopZ1a(C216A)* with deletion of N-terminal 31 aa. Kan^R	Jiang *et al.*, 2013
pRSF::*HopZ1a(K289R)(32-326)*	N-terminal 6×His-SUMO tagged *HopZ1a(K289R)* with deletion of N-terminal 31 aa. Kan^R	Shushu Jiang
pRSF::*HopZ1a(S349AS351A)(32-326)*	N-terminal 6×His-SUMO tagged *HopZ1a(S349AS351A)* with deletion of N-terminal 31 aa. Kan^R	Shushu Jiang
pRSF::*HopZ1a(K289RS349AS351A)(32-326)*	N-terminal 6×His-SUMO tagged *HopZ1a(K289RS349AS351A)* with deletion of N-terminal 31 aa. Kan^R	Shushu Jiang
pRSF::*HopZ1a*	N-terminal 6×His-SUMO tagged *HopZ1a*. Kan^R	Shushu Jiang
pRSF::*HopZ1a(C216A)*	N-terminal 6×His-SUMO tagged *HopZ1a(C216A)*. Kan^R	Shushu Jiang

pRSF::*HopZ1a(K289R)*	N-terminal 6×His-SUMO tagged *HopZ1a(K289R).* Kan^R	Shushu Jiang
pRSF::*HopZ1a(S349AS351A)*	N-terminal 6×His-SUMO tagged *HopZ1a(S349AS351A).* Kan^R	Shushu Jiang
pRSF::*HopZ1a(S344AT346A)*	N-terminal 6×His-SUMO tagged *HopZ1a(S344AT346A).* Kan^R	this study
pRSF::*HopZ1a(T346A)*	N-terminal 6×His-SUMO tagged *HopZ1a(T346A).* Kan^R	this study
pRSF::*HopZ1a(67-369)*	N-terminal 6×His-SUMO tagged *HopZ1a* with deletion of N-terminal 66 aa. Kan^R	Shushu Jiang
pRSF::*HopZ1a(Δ245-281)*	N-terminal 6×His-SUMO tagged *HopZ1a* with deletion of linker 245-281 aa. Kan^R	Zhimin Zhang
pRSF::*HopZ1b (44-368)*	N-terminal 6×His-SUMO tagged *HopZ1b* with deletion of N-terminal 43 aa. Kan^R	Shushu Jiang
pRSF::*HopZ1b(C212A) (44-368)*	N-terminal 6×His-SUMO tagged *HopZ1b(C212A)* with deletion of N-terminal 43 aa. Kan^R	Shushu Jiang
pRSF::*HopZ1b (44-303)*	N-terminal 6×His-SUMO tagged *HopZ1b* with deletion of N-terminal 43 aa and C-terminal 304-368aa. Kan^R	Zhimin Zhang
pRSF::*HopZ1b (44-262)*	N-terminal 6×His-SUMO tagged *HopZ1b* with deletion of N-terminal 43 aa and C-terminal 263-368aa. Kan^R	Zhimin Zhang
pRSF::*HopZ1b(Δ241-270)*	N-terminal 6×His-SUMO tagged *HopZ1b* with deletion of linker 241-270 aa. Kan^R	Zhimin Zhang
pRSF::*PopP2(Δ1-148)*	N-terminal 6×His-SUMO tagged *PopP2* with deletion of N-terminal 148 aa. Kan^R	Shushu Jiang
pRSF::*PopP2(C321A)(Δ1-148)*	N-terminal 6×His-SUMO tagged *PopP2(C321A)* with deletion of N-terminal 148 aa. Kan^R	Shushu Jiang
pRSF::*PopP2(K383A)(Δ1-148)*	N-terminal 6×His-SUMO tagged *PopP2(K383A)* with deletion of N-terminal 148 aa. Kan^R	this study
pRSF::*PopP2(S447AS449A)(Δ1-148)*	N-terminal 6×His-SUMO tagged *PopP2(S337AS449A)* with deletion of N-terminal 148 aa. Kan^R	this study
pET14b::*HopZ3*	N-terminal 6×His tagged *HopZ3*, Amp^R	Ma et al., 2006
pET14b::*HopZ3(C300A)*	N-terminal 6×His tagged *HopZ3* with cys300 replaced with an alanine, Amp^R	Ma et al., 2006
pET14b::*HopZ3(K337A)*	N-terminal 6×His tagged *HopZ3* with lys337 replaced with an alanine, Amp^R	Shushu Jiang
pET14b::*HopZ3(S386A)*	N-terminal 6×His tagged *HopZ3* with ser386 replaced with an alanine, Amp^R	Shushu Jiang

RESULTS

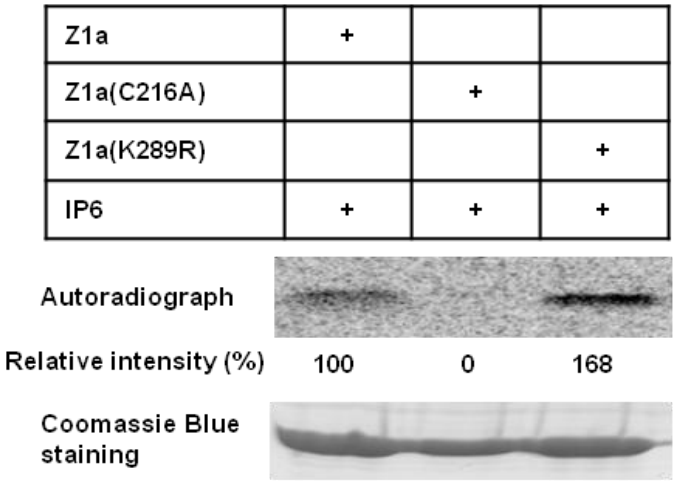
K289 is not an autoacetylation site of HopZ1a

Previously, a conserved lysine residue, K383, in PopP2 was identified as the autoacetylation site (Tasset et al., 2010). The corresponding K289 in HopZ1a was also suggested to be the autoacetylation site as both autoacetylation *in vitro* and biological function in Arabidopsis were lost from GST-HopZ1a(K289R) (Lee et al., 2012). To confirm the role of K289, I purified tag-free HopZ1a(K289R) proteins and examined the autoacetylation activity using an *in vitro* acetylation assay in the presence of C14-labeled acetyl-CoA and the eukaryotic co-factor IP6. Inconsistent with the previous report, loss of autoacetylation was not detected from HopZ1a(K289R) (Figure 1.1). Instead, a stronger autoacetylation signal was consistently detected from this mutant.

Figure 1.1. Autoacetylation activity of HopZ1a(K289R) is not abolished

Tag free HopZ1a(K289R) was subjected to *in vitro* acetylation assay supplemented with 100nM IP6 as a cofactor. Instead of showing a reduction in autoacetylation signal, HopZ1a(K289R) showed a significant higher autoacetylation level than wild type HopZ1a. The acetylated proteins were detected by autoradiography after exposure at -80 ℃ for five days. Numbers underneath the autoradiograph indicate relative acetylation levels of HopZ1a mutants compared to wild-type protein (as 100%). Equal loading of the proteins was confirmed by Coomassie blue staining.

The result was repeated more than three times with consistent results.



Based on the autoacetylation data of HopZ1a(K289R), I speculated that either 1) K289 is not an autoacetylation site of HopZ1a; or 2) K289 is not the only and major autoacetylation site of HopZ1a. In order to gain a clearer picture of the acetylation profile of HopZ1a, I carried out mass spectrometry (MS) analysis and the catalytic mutant HopZ1a(C216A) was used as a negative control. Several potential acetylation sites, most of which are lysine, threonine and serine residues, were identified from both wild-type HopZ1a and the catalytic mutant HopZ1a(C216A) (Figure 1.2). These residues were likely being acetylated independent of the enzymatic activity of HopZ1a, and therefore not considered as candidate autoacetylation sites. I identified potential acetylation for the peptide 289 – 300 amino acid[1], which contains K289. However, further fragmentation analysis revealed that T295, instead of K289, was being acetylated. Moreover, this acetylated peptide was identified in both HopZ1a and HopZ1a(C216A), suggesting that this acetylation is not dependent on the enzymatic activity of HopZ1a. These results confirmed that K289 is not an autoacetylation site of HopZ1a.

[1] The amino acid number denoted here corresponded to the actual residue for full length HopZ1a. Please be noted that an additional serine was left as the first residue of tag-free HopZ1a after cleavage from the SUMO-His tag. For example, amino acid 289-300 denoted here corresponded to amino acid 290 -301 as shown in Figure 1.2.

Figure 1.2. Summary of potential autoacetylation sites in HopZ1a and HopZ1a(C216A) detected by mass spectrometry.

In vitro acetylation using C12 acetyl CoA as acetyl group donor was carried out on HopZ1a and HopZ1a(C216A). Acetylated HopZ1a and HopZ1a(C216A) were then subjected to mass spectrometry analysis. The peptides identified from mass spectrometry are highlighted yellow. Peptides with potential acetylation(s) are underlined and the residues to be acetylated highlighted green. The cysteine to alanine mutation on the catalytic residue C216 is in red.

HopZ1a, 358/370 amino acid (97% coverage)

```
  1 SMGNVCVGGS RMSHQVYSPD RADTPPRSER NTPDRRQRAA GDAERTQSMR
 51 LQQKINDLKP YVRHARGPIK AYGQAALDRA SGKKTSVSFA ELDATHLDAM
101 VYIENQRNPG LNLKHFRDHK ELIQALQSDG PSAFRAIFPQ TCPETGQTLK
151 HHVMADVRLH QGGAPTIIIT EPAVIVGARY QQLQRHNLTL EDLSESGVPL
201 SQVAIIETQA QKTSDDCVMY SLNYAIKAHK NAAQFDDIHH GLQHGTLSTE
251 SESRARTTLG ALEASSSYSV MHEGAHAAFG ADVLPVDFYK HGASLTQAKQ
301 LMKRPDGRMA GRVNSEGHSE AENLVQRNQA FRVKRRELLD DETPSNTQFS
351 ASIDGFRLQE IKRVLAEEQR
```

HopZ1a(C216A), 363/370 amino acid (98% coverage)

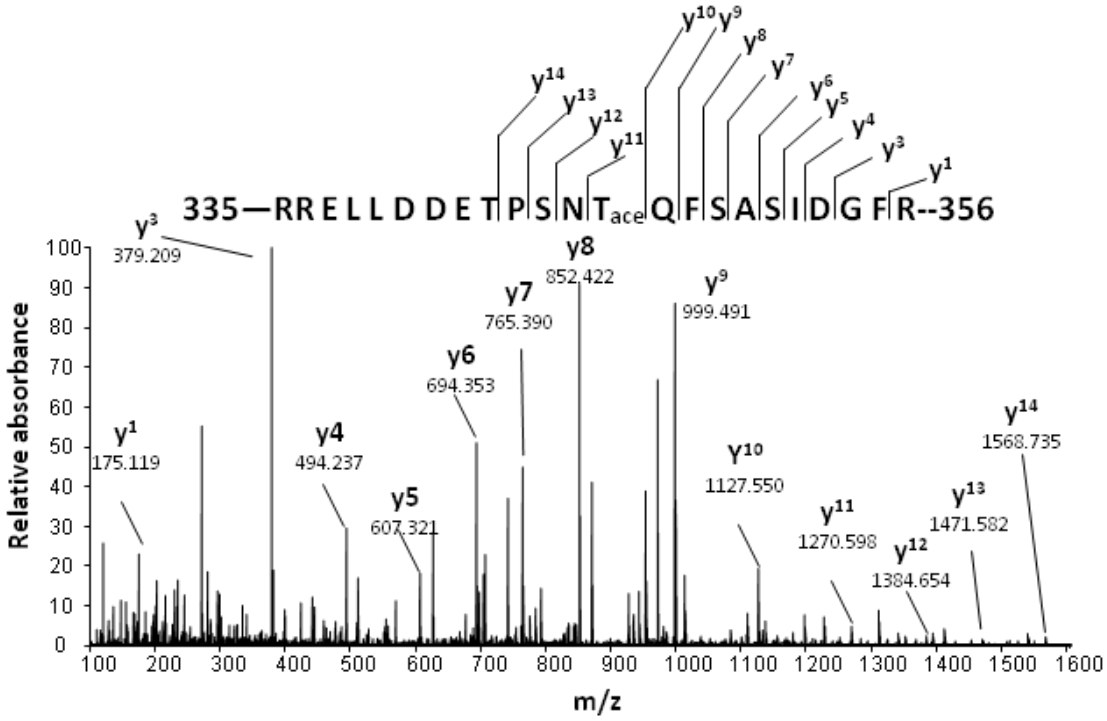
```
  1 SMGNVCVGGS RMSHQVYSPD RADTPPRSER NTPDRRQRAA GDAERTQSMR
 51 LQQKINDLKP YVRHARGPIK AYGQAALDRA SGKKTSVSFA ELDATHLDAM
101 VYIENQRNPG LNLKHFRDHK ELIQALQSDG PSAFRAIFPQ TCPETGQTLK
151 HHVMADVRLH QGGAPTIIIT EPAVIVGARY QQLQRHNLTL EDLSESGVPL
201 SQVAIIETQA QKTSDDAVMY SLNYAIKAHK NAAQFDDIHH GLQHGTLSTE
251 SESRARTTLG ALEASSSYSV MHEGAHAAFG ADVLPVDFYK HGASLTQAKQ
301 LMKRPDGRMA GRVNSEGHSE AENLVQRNQA FRVKRRELLD DETPSNTQFS
351 ASIDGFRLQE IKRVLAEEQR
```

T346 is the predominant autoacetylation site of HopZ1a

From the mass spectrometry analysis of HopZ1a and HopZ1a(C216A), I also identified a peptide spanning 335-356 amino acid showing a mass shift of 42 atomic mass units (amu), possibly due to acetylation, only in wild-type HopZ1a. This peptide therefore likely contains a residue that is acetylated in a HopZ1a acetyltransferase-dependent manner. Fragmentation analysis further identified threonine346 (T346) as the autoacetylation site (Figure 1.3)

Figure 1.3. T346 is autoacetylated in HopZ1a *in vitro*.

Mass spectrometry analysis of HopZ1a peptide spanning amino acid 355-356. Fragmentation of this peptide identified T346 as the residue being acetylated in wild-type HopZ1a, but not HopZ1a(C216A). y designates the C-terminal fragments of the peptide generated from the cleavage of peptide bond in the mass spectrometer (parent ion: *m/z* 847.4025,3+).



Autoacetylation level of HopZ1a(T346A) is similar to wild-type HopZ1a

To further confirm the role of T346, I examined the autoacetylation level of HopZ1a(T346A) *in vitro*. Unexpectedly, HopZ1a(T346A) exhibited a similar level of autoacetylation as wild-type HopZ1a (Figure 1.4). As YopJ family effectors have been shown to acetylate several amino acids including Ser, Thr and Lys (Mukherjee et al., 2006; Trosky et al., 2007; Jones et al., 2008; Tasset et al., 2010). In close proximity to T346, the acetylated tryptic peptide contains additional residues, i.e. T342, S344, S349 and S351, that could potentially be acetylated. I hypothesized that these residues could be acetylated in a complementary manner when T346 is mutated. I therefore systematically mutated these residues and examined these mutants using *in vitro* acetylation assay.

Among the mutants tested, no differences of autoacetylation to wild-type HopZ1a could be detected for the double mutant HopZ1a(S344AT346A) or the single mutant HopZ1a(S349A) and HopZ1a(S351A) (Figure 1.4 & 1.5). However, approximately 70% reduction of the autoacetylation level was detected from the double mutant HopZ1a(S349AS351A) (Figure 1.5).

Figure 1.4. Autoacetylation was not affected significantly when T346 was mutated

Tag free HopZ1a and mutants were subjected to *in vitro* acetylation assay. IP6 was supplemented with as a cofactor. No significant difference of autoacetylation level could be detected from HopZ1a(T346A) to wild type HopZ1a. Simultaneous mutation of T346 and the nearby S344 did not affect autoacetylation either. The acetylated proteins were detected by autoradiography after exposure at -80 ℃ for five days. Numbers underneath the autoradiograph indicate relative acetylation levels of HopZ1a mutants compared to wild-type protein (as 100%). Equal loading of the proteins was confirmed by Coomassie blue staining.

The result was repeated twice with consistent results.

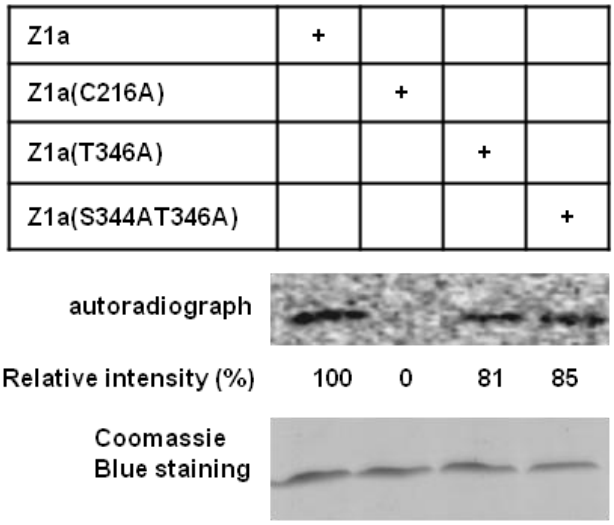
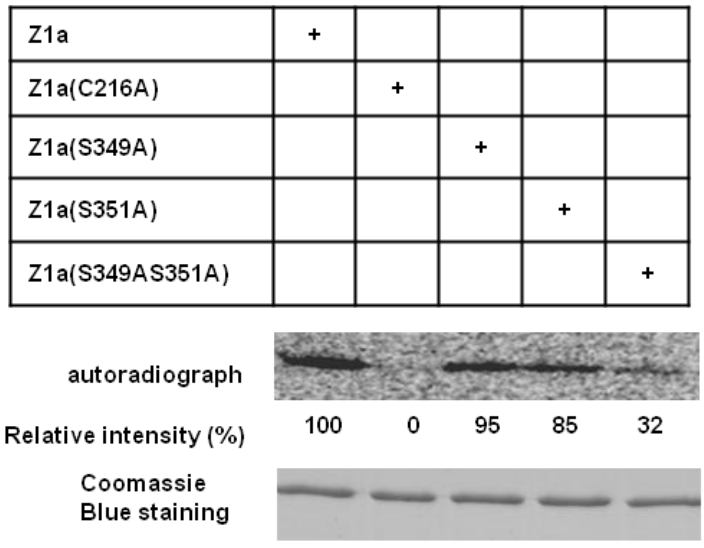


Figure 1.5. HopZ1a(S349AS351A) exhibited significant reduction in autoacetylation.

Tag free HopZ1a(S349A), HopZ1a(S351A) and HopZ1a(S349AS351A) were subjected to *in vitro* acetylation assay. IP6 was supplemented as a cofactor. No significant difference of autoacetylation level could be detected from the single mutant of HopZ1a(S349A) and HopZ1a(S351A). About 70% reduction of acetylation level was detected from HopZ1a(S349AS351A). The acetylated proteins were detected by autoradiography after exposure at -80 ℃ for five days. Numbers underneath the autoradiograph indicate relative acetylation levels of HopZ1a mutants compared to wild-type protein (as 100%). Equal loading of the proteins was confirmed by Coomassie blue staining.

The result was repeated twice with consistent results.



Acetylation profile of HopZ1a changes when T346 is absent

Since the autoacetylation level of HopZ1a(T346A) was unchanged compared to wild-type HopZ1a and the double mutant HopZ1a(S349AS351A) showed a significant decrease of acetylation, I hypothesized that residue S349 and S351 may be acetylated when T346 was not available. To test this hypothesis, additional mass spectrometry analysis of HopZ1a(T346A) was carried out. This experiment revealed that T342 could be acetylated when T346 was absent. No acetylations could be detected for S349 and S351. Several acetylation sites identified from the previous experiment on HopZ1a and HopZ1a(C216A) were not detected and new acetylation sites appeared (Figure 1.6). This data suggested that the acetylation profile of HopZ1a changed when T346 was not available.

Figure 1.6. Mass spectrometry analysis of additional autoacetylation sites in HopZ1a(T346A)

HopZ1a(T346A) was acetylated *in vitro* and subjected to mass spectrometry analysis in the same way as HopZ1a and HopZ1a(C216A). No acetylation could be detected on S349 and S351. Instead, T342 was acetylated in HopZ1a(T346A) background. The peptides identified from mass spectrometry are highlighted yellow. Peptides with potential acetylation(s) are underlined and the residues to be acetylated highlighted green. The catalytic reside C216 is in red and the mutation on T346 is in blue. The summary of the acetylation profile of HopZ1a from Figure 1.2 is included for direct comparison.

HopZ1a(T346A), 316/370 amino acid (85.4% coverage)

```
  1 SMGNVCVGGS RMSHQVYSPD RADTPPRSER NTPDRRQRAA GDAERTQSMR
 51 LQQKINDLKP YVRHARGPIK AYGQAALDRA SGKKTSVSFA ELDATHLDAM
101 VYIENQRNPG LNLKHFRDHK ELIQALQSDG PSAFRAIFPQ TCPETGQTLK
151 HHVMADVRLH QGGAPTIIIT EPAVIVGARY QQLQRHNLTL EDLSESGVPL
201 SQVAIIETQA QKTSDDCVMY SLNYAIKAHK NAAQFDDIHH GLQHGTLSTE
251 SESRARTTLG ALEASSSYSV MHEGAHAAFG ADVLPVDFYK HGASLTQAKQ
301 LMKRPDGRMA GRVNSEGHSE AENLVQRNQA FRVKRRELLD DETPSNAQFS
351 ASIDGFRLQE IKRVLAEEQR
```

HopZ1a, 358/370 amino acid (97% coverage) (from Figure 1.2)

```
  1 SMGNVCVGGS RMSHQVYSPD RADTPPRSER NTPDRRQRAA GDAERTQSMR
 51 LQQKINDLKP YVRHARGPIK AYGQAALDRA SGKKTSVSFA ELDATHLDAM
101 VYIENQRNPG LNLKHFRDHK ELIQALQSDG PSAFRAIFPQ TCPETGQTLK
151 HHVMADVRLH QGGAPTIIIT EPAVIVGARY QQLQRHNLTL EDLSESGVPL
201 SQVAIIETQA QKTSDDCVMY SLNYAIKAHK NAAQFDDIHH GLQHGTLSTE
251 SESRARTTLG ALEASSSYSV MHEGAHAAFG ADVLPVDFYK HGASLTQAKQ
301 LMKRPDGRMA GRVNSEGHSE AENLVQRNQA FRVKRRELLD DETPSNTQFS
351 ASIDGFRLQE IKRVLAEEQR
```

S349 and S351 are required for host recognition of HopZ1a

Based on the results from the acetylation assays, I hypothesized that S349 and S351 would also be required for HopZ1a function in plants. In *Arabidopsis thaliana* eco. Col-0, HopZ1a elicits hypersensitive response (HR) depending on the R protein ZAR1 (Lewis et al., 2010). Furthermore, the catalytic mutant HopZ1a(C216A) is abrogated for this HR-triggering activity (Ma et al., 2006). In order to test whether HopZ1a(S349AS351A) still retained the ability to elicit HR in Arabidopsis, I introduced wild-type or mutant *HopZ1a* genes into *Pseudomonas syringae* pv. *tomato* strain D28E (*Pto*D28E), a mutant of wild-type strain DC3000 with 28 endogenous type III effectors deleted (Cunnac et al., 2011). *Pto*D28E still carry a functional type III secretion apparatus, allowing the delivery of HopZ1a or the mutants into plant cells; however, in the absence of the endogenous type III effectors, the HR-triggering phenotype by HopZ1a would not be interfered. Bacterial infiltration assays showed that *Pto*D28E expressing the mutant HopZ1a(S349AS351A) was no longer able to induce cell death in Arabidopsis eco. Col-0 (Figure 1.7). Consistent with the *in vitro* acetylation data, mutants including HopZ1a(T346A), Z1a(S349A) and Z1a(S351A), which exhibited unaltered acetylation activity to wild type HopZ1a, were still able to trigger HR (Figure 1.7). The observed changes in defense-eliciting activity of HopZ1a mutants were not due to the altered expression levels of the mutant proteins as the expression of mutant proteins were confirmed by western blots (Figure 1.8).

HR was not detected in soybean upon infiltration of *P. syringae* pv. *glycinea* BR1-O1 expressing HopZ1a(S349AS351A) (Figure 1.9), suggesting that S349 and S351 are required for HopZ1a to elicit HR in Arabidopsis and soybean. However, unlike the

catalytic mutant HopZ1a(C216A), cell death was still elicited by HopZ1a(K289R) and HopZ1a(S349AS351A) in *Nicotiana benthamiana*, suggesting that HopZ1a(K289R) and HopZ1a(S349AS351A) may still carry a function in *N. benthamiana* (Figure 1.10).

Figure 1.7. S349 and S351 are required for HopZ1a recognition in Arabidopsis ecotype Col-0

Substitutions of S349 and S351 abolished the cell death triggered by HopZ1a in Arabidopsis. *Pto*D28E expressing wild-type or mutant HopZ1a were infiltrated into leaves of five-week old Arabidopsis eco. Col-0 plants at OD_{600} = 0.2 ($2x10^8$ cfu/mL). Cell death symptoms were monitored at 24 hours post inoculation (hpi). *Pto*DC3000 carrying the empty vector pUCP20tk (EV) and HopZ1a(C216A) were used as a negative control. The experiment was repeated twice with consistent results.


EV
Z1a
Z1a(C216A)
Z1a(K289R)
Z1a(S344A)
Z1a(T346A)
Z1a(S344AT346A)
Z1a(S349A)
Z1a(S351A)
Z1a(S349A S351A)
Z1a(K289R S349AS351A)

Figure 1.8. Expression of HopZ1a and mutants in PtoD28E transformants.

Immunoblot showing the expression of wild-type and mutant HopZ1a in strain *Pto*D28E. The mutant *HopZ1a* genes, tagged with HA, were expressed under its native promoter and carried in the plasmid vector pUCP20tk. Proteins were induced in M63 minimal medium containing 1% fructose overnight at room temperature. Expression of HopZ1a proteins in induced bacterial cells was detected by anti-HA antibody (Roche Diagnostics). Equal loading of the proteins was confirmed by Coomassie blue staining.

The experiment was repeated once with consistent result.

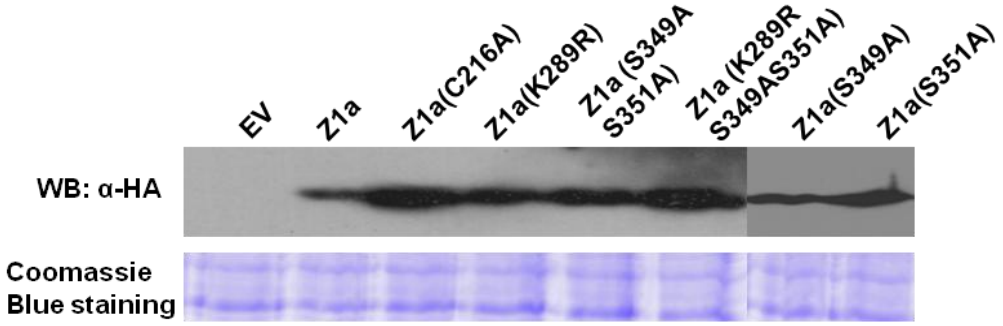


Figure 1.9. S349 and S351 are required to elicit HR in soybean William 82

Substitutions of K289, S349 and S351 abolished the cell death triggered by HopZ1a in soybean Williams 82. *Pgy*BR1-O1 expressing wild-type or mutant HopZ1a were infiltrated into leaves of two-week old soybean plants at OD_{600} = 0.1 (approximately 1×10^8 cfu/mL). Cell death symptoms were monitored at 36-48 hours post inoculation (hpi). HopZ1a(C216A) was used as a negative control.

The experiment was repeated twice with consistent results.

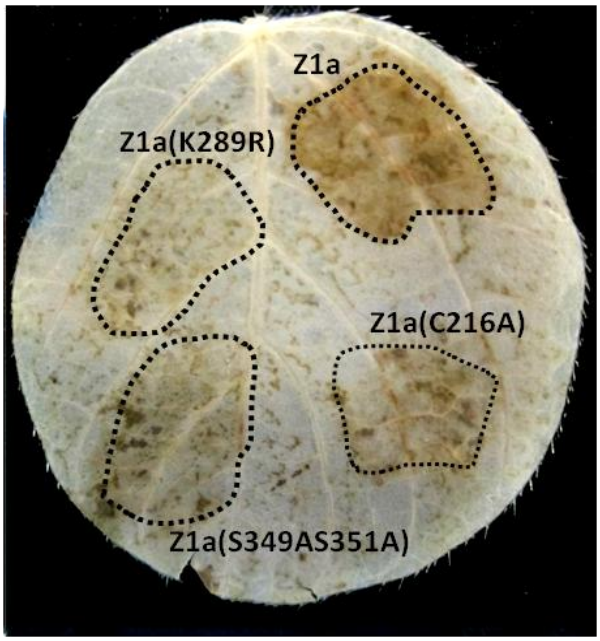
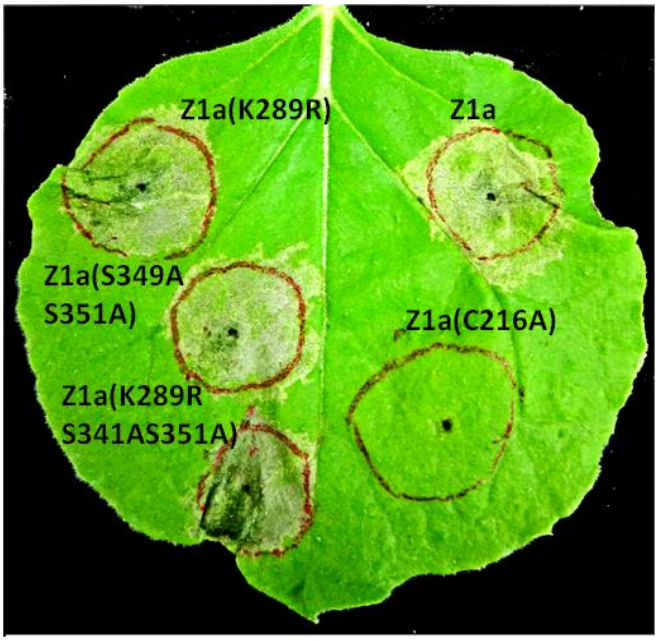


Figure 1.10. HopZ1a(S349AS351A) still elicits cell death in *N. benthamiana.*

Mutations on K289, S349 and S351 do not affect HopZ1a-mediated cell death in *N. benthamiana*. *Agrobacterium tumefaciens* (C58C1) expressing wild-type or mutant HopZ1a were infiltrated into leaves of three-week old *N. benthamiana* at final OD_{600} = 0.05 (approximately 5×10^7 cfu/mL). Agrobacterium expressing the silencing suppressor P19 was co-infiltrated together with the HopZ1a constructs. Cell death symptoms were monitored at 24 hours post inoculation (hpi). HopZ1a(C216A) was used as a negative control.

The experiment was repeated twice with consistent results.

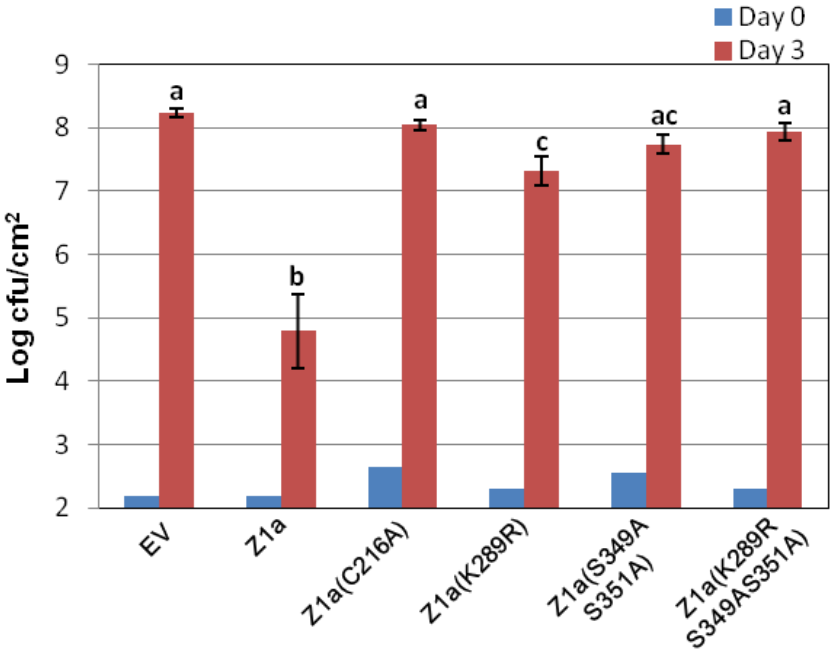


To provide a more quantitative measurement on the contribution of S349 and S351 to HopZ1a-triggered defense response, I determined the bacterial population of *Pto*DC3000 expressing wild-type or mutant forms of HopZ1a in Arabidopsis Col-0. Consistent with the loss of cell death phenotype (Figure 1.7), *Pto*DC3000 expressing HopZ1a(S349AS351A) multiplied to a similar level as *Pto*DC3000 carrying the empty vector (EV) or expressing the catalytic mutant HopZ1a(C216A) (Figure 1.11). Although HopZ1a(K289R) was unable to elicit cell death in the infiltrated leaves (Figure 1.7), the population of *Pto*DC3000(HopZ1a(K289R)) was about 5-10 folds lower than those of *Pto*DC3000(HopZ1a(S349AS351A)) or *Pto*DC3000(HopZ1a(C216A)) (Figure 1.11). This data suggest that HopZ1a(K289R) could still elicit a weak defense response while HopZ1a(S349AS351A) lost most of its activity in Arabidopsis. The triple mutant HopZ1a(K289RS349AS351A) lost the HR-triggering activity completely.

Figure 1.11. Bacterial multiplications of *Pto*DC3000 expressing HopZ1a or its mutants.

HopZ1a(S349AS351A) does not elicit strong defense response in Arabidopsis eco. Col-0 while HopZ1a(K289R) still elicit a weak defense response. Mutations of K289, S349 and S351 rendered HopZ1a losing the ability to trigger defense similar to HopZ1a(C216A). Five-week old Arabidopsis leaves were infiltrated with *Pto*DC3000 carrying pUCP20tk (EV), or expressing wild-type or mutant HopZ1a at OD_{600} = 0.0001 ($1x10^5$ cfu/mL). Bacterial populations were determined at 0 and 3 days post inoculation (dpi). The average colony forming units per square centimeter (cfu/cm^2) and standard deviations (as error bars) are presented. Different letters on top of the bars represent data with statistically significant differences (two tailed t-test $p<0.05$).

The experiment was repeated three times with similar results.



S349 and S351 are required for the virulence activities of HopZ1a

In the absence of the R gene *zar1,* HopZ1a promotes *P. syringae* infection in Arabidopsis (Jiang et al., 2013) and inhibits plant cell wall callose deposition induced by flg22, the major PAMP of *P. syringae* (Lee et al., 2012). After confirming the requirement of S349 and S351 for the avirulence function of HopZ1a, I next examined the contribution of S349 and S351 to the virulence activities of HopZ1a.

Callose is an amorphous β-(1,3)-glucan polymer that reinforces cell wall in the form of papillae as a physical barrier to slow pathogen infection. Callose deposition is triggered by perception of PAMP including flg22 during pathogen infection and is commonly used as an output of early PTI response (Luna et al., 2011). In order to rule out the effect of HR in interfering with the virulence function of HopZ1a in Arabidopsis, my colleague Dr. Shushu Jiang transformed Arabidopsis plants expressing wild-type or mutants HopZ1a. These transgenic plants were generated in the *zar1-1* background such that the virulence function of HopZ1a would not be masked over by the strong HR.

HopZ1a and HopZ1a mutant transgenic plants were infiltrated with 1μM flg22 and the numbers of callose were numerated. I showed that HopZ1a(K289R) and HopZ1a(S349AS351A) lost the ability to suppress callose deposition triggered by flg22, similar to the catalytic mutant HopZ1a(C216A) (Figure 1.12). The loss of callose deposition suppression activity was not due to a lower expression of HopZ1a mutants as similar expression of HopZ1a in transgenic Arabidopsis *zar1-1* were confirmed by western blotting (Figure 1.13).

Figure 1.12. S349 and S351 are required for HopZ1a to suppress callose deposition

Mutations on S349 and S351 abolished the ability of HopZ1a to suppress flg22-mediated callose deposition. Adult leaves of five-week old transgenic Arabidopsis *zar1-1* plants expressing wild-type or mutant HopZ1a were infiltrated with 1 μM of flg22. The infiltrated leaves were collected at 16 hours post infiltration and stained with aniline blue (MP Biomedicals, Santa Ana, CA). Numbers of callose in the infiltrated areas were numerated using a florescent microscopy under UV. Values are means ± standard deviations (n=12).

The experiment was repeated twice with consistent results.

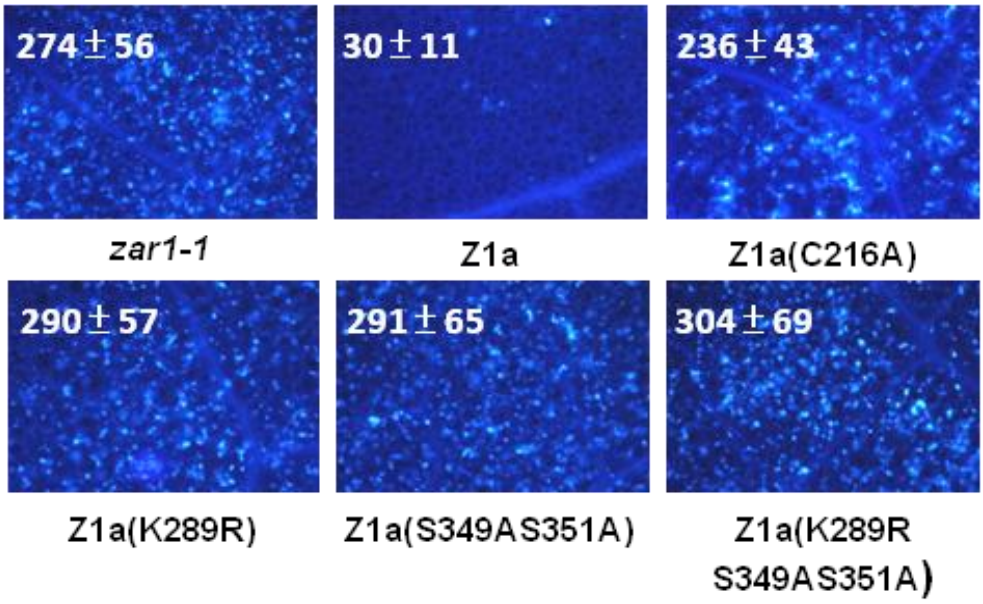
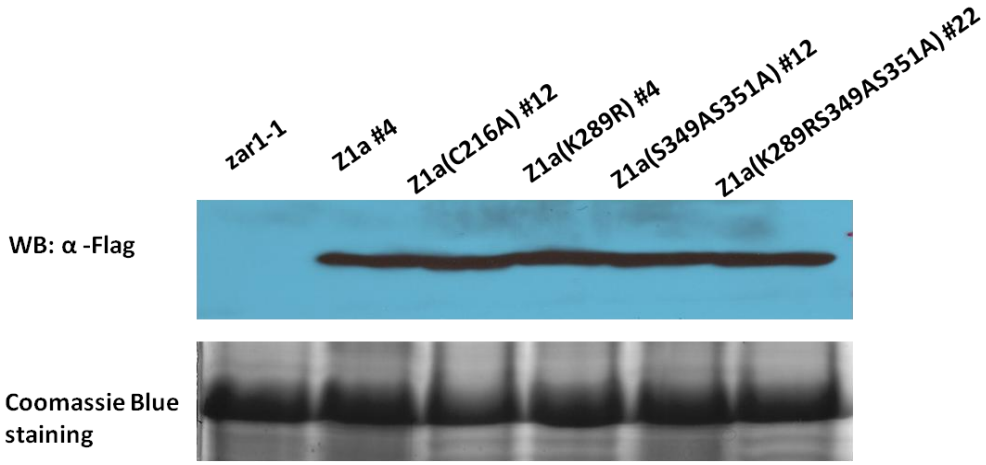


Figure 1.13. Expression of HopZ1a and mutants in transgenic Arabidopsis.

3XFlag-HopZ1a and mutant genes, under the control of Cauliflower mosaic virus *35S* constitutive promoter were cloned into the vector pEG100 and transformed into *zar1-1* Arabidopsis plants. The expression of HopZ1a and mutants were detected by anti-Flag antibody (Santa Cruz Biotechnology). Equal loading of the proteins was confirmed by Coomassie blue staining.

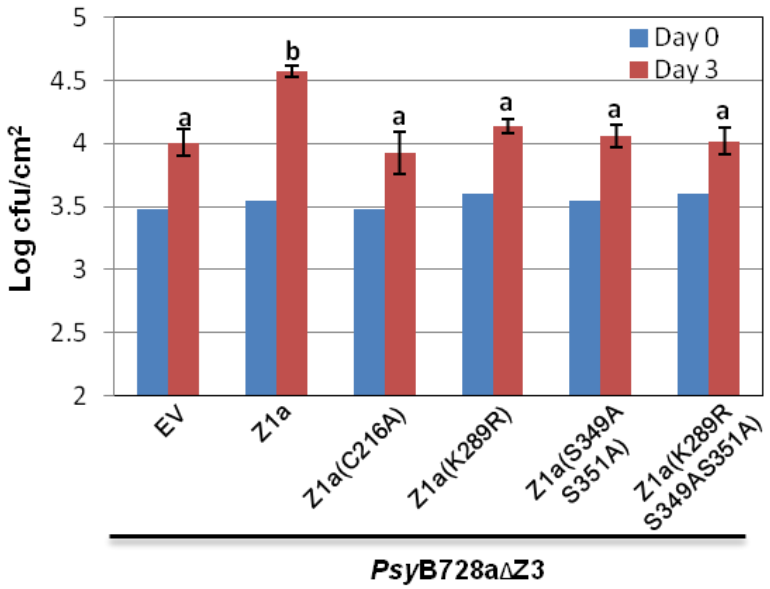


To further confirm that S349 and S351 are required for HopZ1a to promote bacterial infection, I introduced wild-type and mutants of HopZ1a into *Pseudomonas syringae* pv. *syringae* (*Psy*) strain B728aΔ*Z3* (Vinatzer et al., 2006) and evaluated the bacterial populations in Arabidopsis *zar1-1* plants. *Psy*B728aΔ*Z3* is a non-pathogen of Arabidopsis Col-0 and thus serves as a useful tool to evaluate virulence activity of HopZ1a. Expression of HopZ1a promoted bacterial infection and the population of *Psy*B728aΔ*Z3*(HopZ1a) was fivefold greater than *Psy*B728aΔZ3. This growth promotion activity was lost in the mutants HopZ1a(S349AS351A), HopZ1a(K289R) or HopZ1a(K289RS349AS351A) (Figure 1.14) .

Figure 1.14. S349 and S351 are required for HopZ1a to promote bacterial multiplication of *Psy*B728aΔZ3.

HopZ1a(S349AS351A) cannot promote growth of *Psy*B728aΔZ3. Five-week old Arabidopsis leaves were infiltrated with *Psy*B728aΔZ3 carrying pUCP20tk (EV), or expressing wild-type or mutant HopZ1a at OD_{600} = 0.005 ($5x10^6$ cfu/mL). Bacterial populations were determined at 0 and 3 days post inoculation (dpi). 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 $p<0.05$).

The experiment was repeated three times with consistent results.

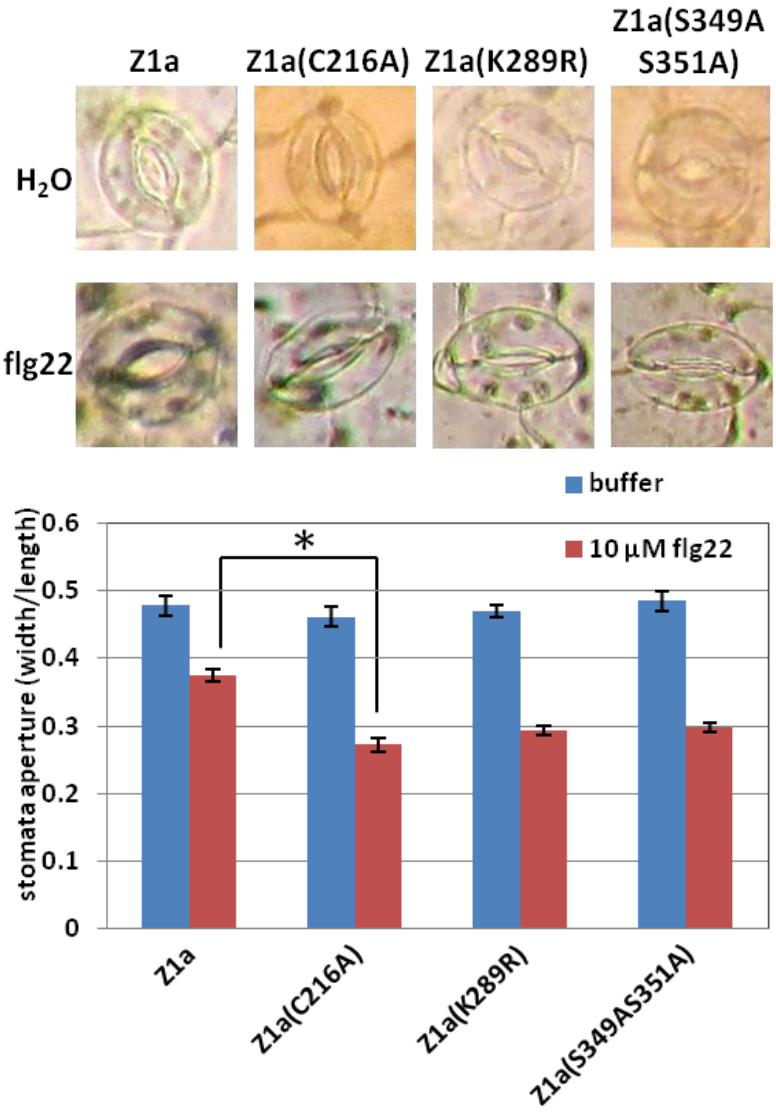


S349 and S351 are required for HopZ1a to suppress stomatal closure

Bacterial infection induces closure of plant stomata as a defense response to prevent bacterial entry. In *P. syringae*, T3SEs HopM1 (Lozano-Duran et al., 2014) and HopF2 (Hurley et al., 2014), as well as the phytotoxin coronatine (COR) (Melotto et al., 2006), can promote infection by interfering with stomatal defense. In particular, COR induces the degradation of JAZ proteins and activates jasmonate (JA) signaling, thus leading to reopening of stomata to facilitate bacterial entry (Melotto et al., 2006; Zheng et al., 2012). HopZ1a is also able to activate JA signaling by targeting the JAZ proteins (Jiang et al., 2013); however, whether HopZ1a could manipulate stomata opening is unknown.

To examine whether HopZ1a is able to suppress stomatal defense, I measured the stomatal aperture in leaves of transgenic Arabidopsis *zar1-1* expressing wild-type or mutants HopZ1a after infiltration with flg22, which induces stomatal closure (Melotto et al., 2006). Two hours after flg22 treatment, the width/length ratio of each stomata (n=60) in the leaves was determined. Consistent with the JA-activating ability of HopZ1a, expression of HopZ1a led to wider stomata aperture in the leaves (Figure 1.15). On the contrary, the mutants HopZ1a(S349AS351A), HopZ1a(K289R) or the catalytic mutant HopZ1a(C216A) were unable to increase stomata aperture (Figure 1.15). These results demonstrate that HopZ1a can suppress stomatal defense in an enzymatic activity-dependent manner and S349 and S351 are required for this virulence activity, probably due to its essential role in the acetyltransferase activity of HopZ1a.

Figure 1.15. S349 and S351 are required to inhibit flg22-induced stomatal closure. Leave disc of four-week old transgenic Arabidopsis *zar1-1* plants expressing wild-type or mutant HopZ1a were incubated with 10 μM of flg22. Two hours after flg22 treatment, stomata on the lower epidermis were mounted on a slide coated with a layer of adhesive (Hollister, Libertyville IL). The stomata were observed under a light microscope. Micrographs of stomata were taken (representative ones were shown in the upper panel) and used to measure the stomata aperture, which is expressed as the ratio of width over length. The average stomatal aperture and the standard error (as error bars) are presented (lower panel). Only wild type HopZ1a suppressed flg22-mediated stomatal closure. Arabidopsis *zar1-1* expressing HopZ1a(C216A) was used as a negative control. The asterisk labels data with statistically significant differences (two tailed t-test $p<0.05$). The experiment was repeated twice with similar results.


Z1a
Z1a(C216A)
Z1a(K289R)
Z1a(S349A S351A)
H2O
flg22
buffer
10 µM flg22
*
stomata aperture (width/length)
0
0.1
0.2
0.3
0.4
0.5
0.6
Z1a
Z1a(C216A)
Z1a(K289R)
Z1a(S349AS351A)

S349 and S351 are required for the interaction with the co-factor IP6

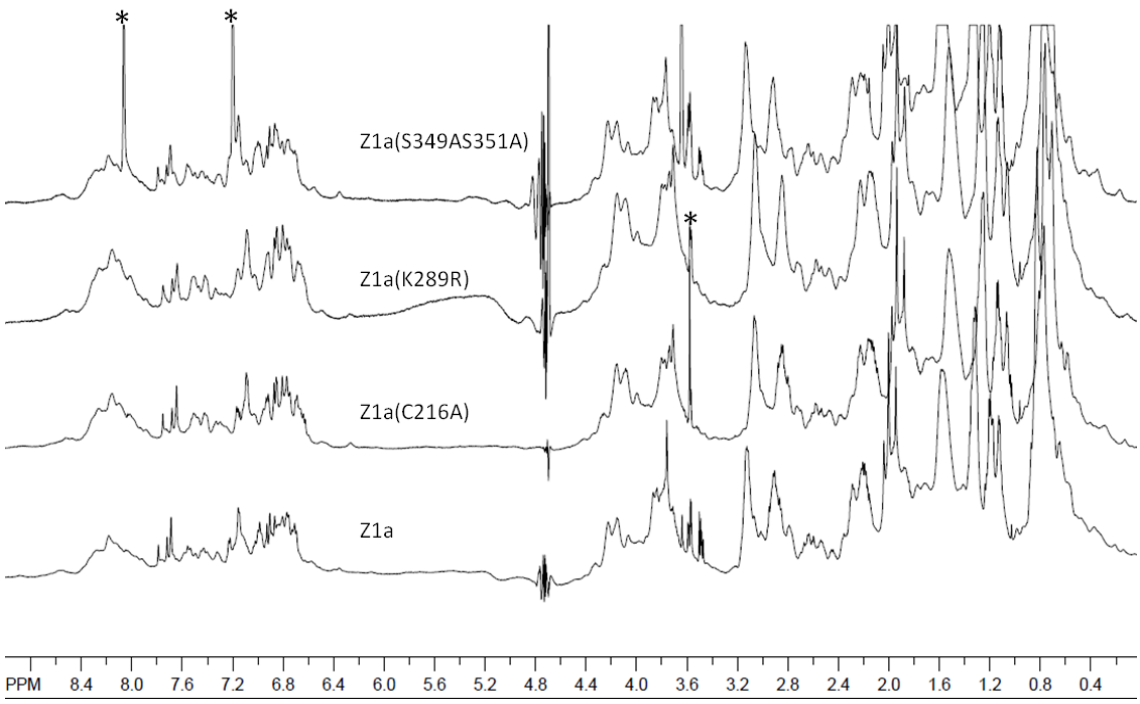
Even though mass spectrometry did not identify K289, S349 and S351 as autoacetylation sites *in vitro*, these residues are indeed required for the enzymatic activity and biological function of HopZ1a. One possibility is that these residues are critical for the structure of HopZ1a. To test this hypothesis, I compared the conformations of HopZ1a, HopZ1a(C216A), HopZ1a(K289R) and HopZ1a(S349AS351A) in solution using 1D proton nuclear magnetic resonance (NMR), which is a powerful analytical tool widely used to characterize the structural and dynamic behaviors of proteins in solution state. NMR is based on the principle that nuclei with unpaired spin including proton (^{1}H) absorbs and radiates electromagnetic radiation under a magnetic field. The resonance frequency of a nuclide in turn depends on the chemical environment such that a different NMR spectrum is observed if the chemical environment changes due to direct residue-ligand interaction or conformation change of the protein itself. The ^{1}H chemical shifts are highly sensitive to local changes of chemical environment, which offer an important parameter to evaluate the protein conformational states and protein-ligand interactions. In general, NMR signals in the aromatic region serve as a good indicator of the folding status of a protein (Pruitt et al., 2009).

The NMR spectra of purified HopZ1a, HopZ1a(C216A), HopZ1a(K289R) and HopZ1a(S349AS351A) proteins did not exhibit significant differences, suggesting that the substitutions of K289, S349 and S351 did not affect the overall conformation of HopZ1a (Figure 1.16).

Figure 1.16. The conformation of HopZ1a is not affected by mutation of K289, S349 and S351.

0.1mM of tag-free wild-type HopZ1a, HopZ1a(C216A), HopZ1a(K289R) and HopZ1a(S349AS351A) were subjected to 1D proton (^{1}H) NMR analysis. 1D proton NMR spectra were collected for HopZ1a and mutant proteins on a Bruker Advance 600 MHz NMR spectrometer at 25 ℃. The NMR spectra were then processed and analyzed using the Topsin software (Bruker Inc., Billerica, MA). The NMR spectra of HopZ1a, HopZ1a(C216A), HopZ1a(K289R) and HopZ1a(S349AS351A) were similar in the absence of cofactor IP6. Peaks labeled by asterisks corresponded to residual hydrogen containing reagents e.g. glycerol (around 3.6 ppm) and Tris (around 7.0 and 8.0 ppm) that do not interfere with the overall NMR analysis of HopZ1a.

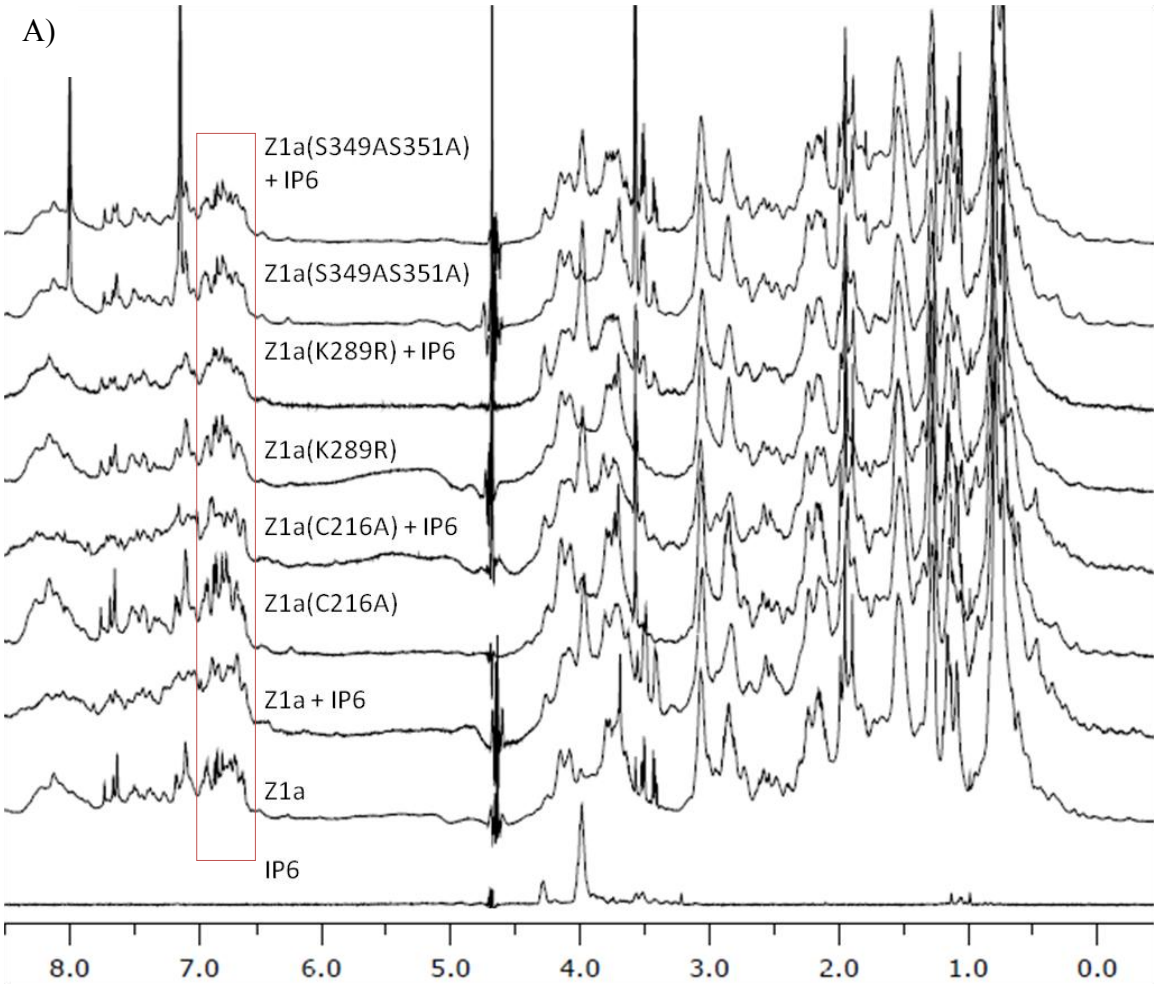
Information of NMR spectra of common chemical agents can be found in Biological Magnetic Resonance Data Bank (http://bmrb.wisc.edu).


Z1a(S349AS351A)
Z1a(K289R)
Z1a(C216A)
Z1a
PPM
8.4
8.0
7.6
7.2
6.8
6.4
6.0
5.6
5.2
4.8
4.4
4.0
3.6
3.2
2.8
2.4
2.0
1.6
1.2
0.8
0.4

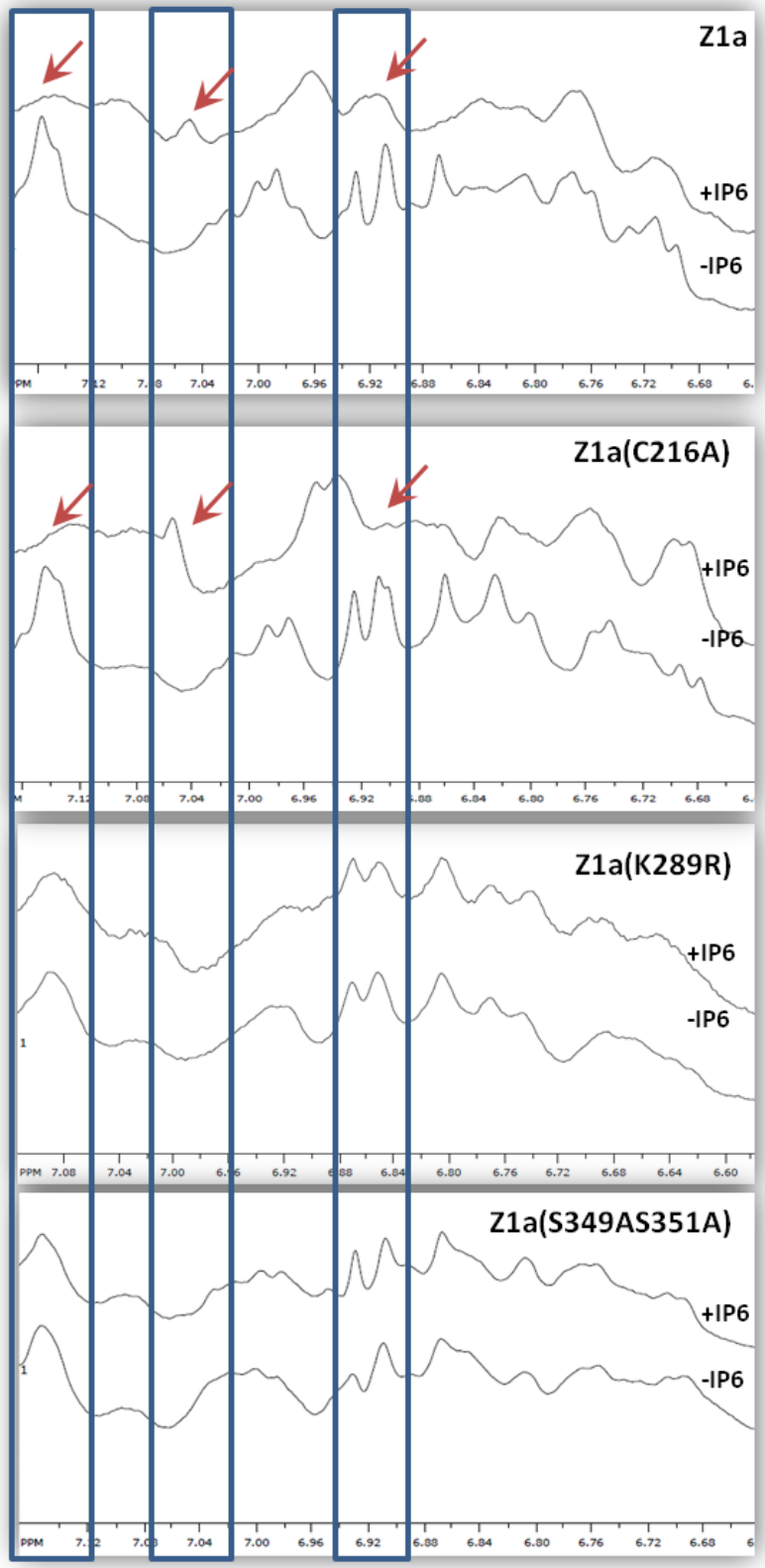
Since extensive conformational change was observed in other YopJ family effectors i.e. AvrA, I examined whether the addition of the cofactor IP6 also induced a conformational change for HopZ1a. Extensive changes in NMR spectrum of HopZ1a was observed in the presence of the co-factor IP6. Addition of IP6 to HopZ1a resulted in a much wider dispersion of NMR signals and peak shifts in the aromatic region (Figure 1.17). These chemical shift changes, together with the emergence of both high frequency and low frequency NMR signals, indicated that IP6 bound to HopZ1a to induce a more compact folding state of HopZ1a. Such an IP6-induced conformational change is independent of the catalytic residue, as the same spectral changes were observed from the catalytic mutant HopZ1a(C216A) in the presence of IP6. By contrast, NMR spectrum of HopZ1a(K289R) and HopZ1a(S349AS351A) was not altered by IP6 (Figure 1.17), suggesting that K289, S349 and S351 are required for the conformational changes of HopZ1a induced by IP6, presumably from an inactivated state to a more compact, activated state.

Figure 1.17. K289, S349 and S351 are required for IP6 mediated conformational change of HopZ1a.

0.1mM HopZ1a and mutant proteins were subjected to 1D proton (^{1}H) NMR analysis. 1mM IP6 (HopZ1a:IP6=1:10) was added to observe for any changes in HopZ1a conformation. A) Perturbation of dispersions was observed in the spectra of both HopZ1a and HopZ1a(C216A) upon addition of IP6. Changes of signals at both high frequency and low frequency were observed in HopZ1 and HopZ1a(C216A), but not in the spectra of HopZ1a(K289R) and HopZ1a(S349AS351A). Dispersions of signals were more prominent in the aromatic region (ppm 6.0-8.5). B) A region with changes (ppm 6.5-7.0) was boxed and amplified for better comparison.


A)
Z1a(S349AS351A) + IP6
Z1a(S349AS351A)
Z1a(K289R) + IP6
Z1a(K289R)
Z1a(C216A) + IP6
Z1a(C216A)
Z1a + IP6
Z1a
IP6
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0

B)


Z1a
+IP6
-IP6
Z1a(C216A)
+IP6
-IP6
Z1a(K289R)
+IP6
-IP6
Z1a(S349AS351A)
+IP6
-IP6

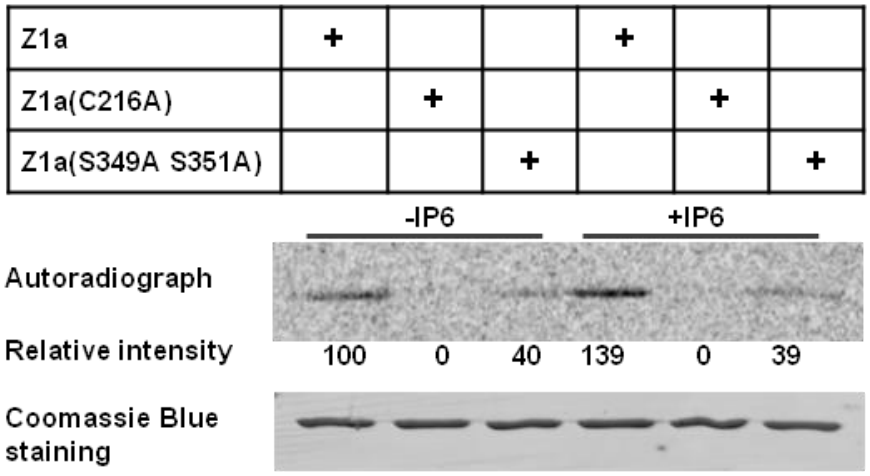
S349 and S351 are required for the IP6-mediated activation of HopZ1a activity

To further confirm that S349 and S351 are essential for IP6-mediated activation of HopZ1a enzymatic activity, I conducted *in vitro* acetylation assays and compared the autoacetylation levels of HopZ1a (S349AS351A) in the presence or absence of IP6. Interestingly, although the autoacetylation level of wild-type HopZ1a was enhanced approximately 40% in the presence of IP6, such an enhancement was not observed for HopZ1a(S349AS351A) (Figure 1.18). These data support a model that S349 and S351 affect HopZ1a enzymatic activity at least partially through interaction with IP6.

Figure 1.18. S349 and S351 are required for IP6-mediated activation of HopZ1a acetyltransferase activity.

In vitro acetylation assays of HopZ1a, HopZ1a(C216A) and HopZ1a(S349AS351A) were carried out in the presence or absence of IP6. Numbers underneath the autoradiograph indicates relative acetylation levels of HopZ1a mutants compared to wild-type protein in the absence of IP6 (as 100%). Equal loading of the proteins was confirmed by Coomassie blue staining.

The experiment was repeated twice with similar results.



Conserved serine residues in other YopJ family effectors are required for their acetyltransferase activity

Sequence alignment revealed that S349 and S351 are conserved in most YopJ family effectors (Figure 1.19). Therefore, I speculated that they may also be important for the enzymatic activity of other YopJ family members. To test this hypothesis, I constructed mutants on the corresponding serine residue(s) in two YopJ family effectors, PopP2 of *Ralstonia solanacearum* and HopZ3 of *P. syringae* pv. *syringae*, and examined their autoacetylation *in vitro*. PopP2 contains both serines at sites 447 and 449 and the mutant PopP2(S447AS449A) is no longer autoacetylated (Figure 1.20). HopZ3 is a YopJ family effector that is also produced by *P. syringae* pv. *syringae* and evolutionarily related to HopZ1a (Ma et al., 2006). I showed that HopZ3 possessed an acetyltransferase activity *in vitro* and mutation of the conserved serine at the site 386 corresponding to S351 of HopZ1a also abolished HopZ3 acetyltransferase activity (Figure 1.20). These data suggest that the serine residue(s) plays a conserved role in YopJ family effectors and are required for their acetyltransferase activities.

Figure 1.19. Sequence alignment of YopJ family effectors showing the region containing the conserved serine residues.

Amino acids sequence alignment in a conserved C-terminal region spanning residue 344-361 of HopZ1a and the corresponding region of other YopJ family effectors are shown. The accession numbers of each protein are presented in parentheses after the species name. Multiple sequence alignment was generated using ClustalX. Positively charged residues are highlighted as red and polar residue as green. Pictogram showing relative frequency of amino acid occurrence at each position is showed above the alignment. S349 and S351 of HopZ1a are labeled by asterisks.

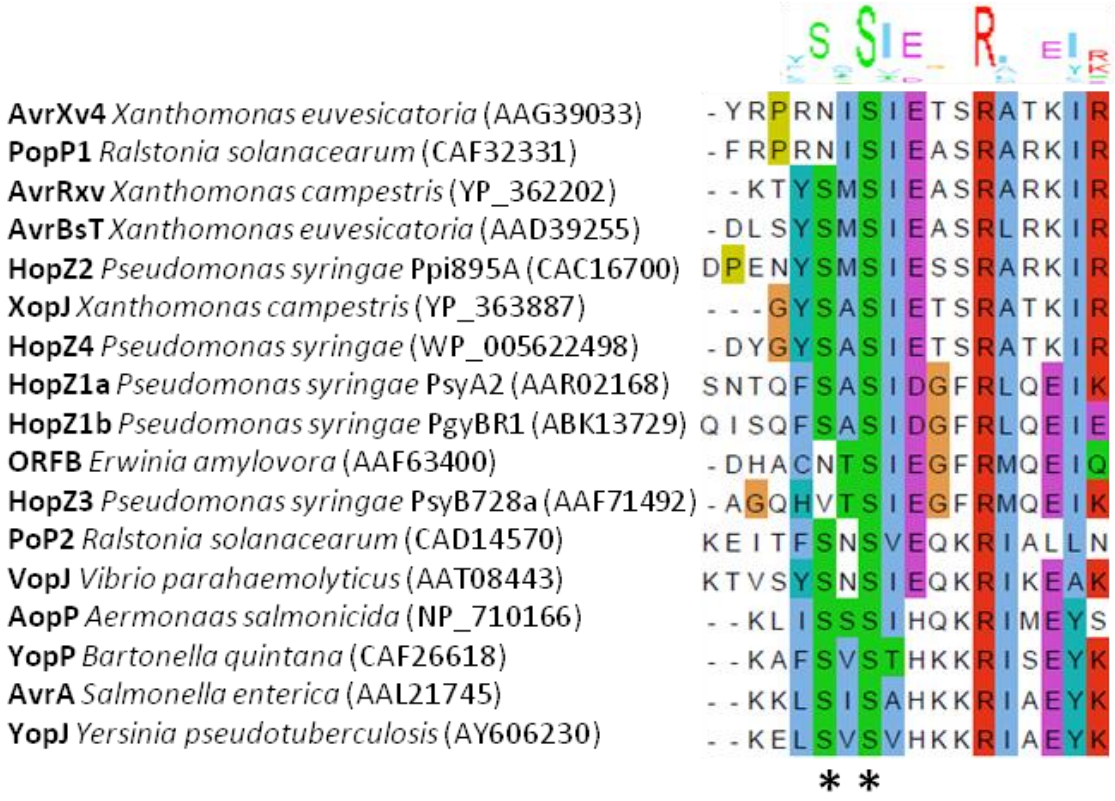
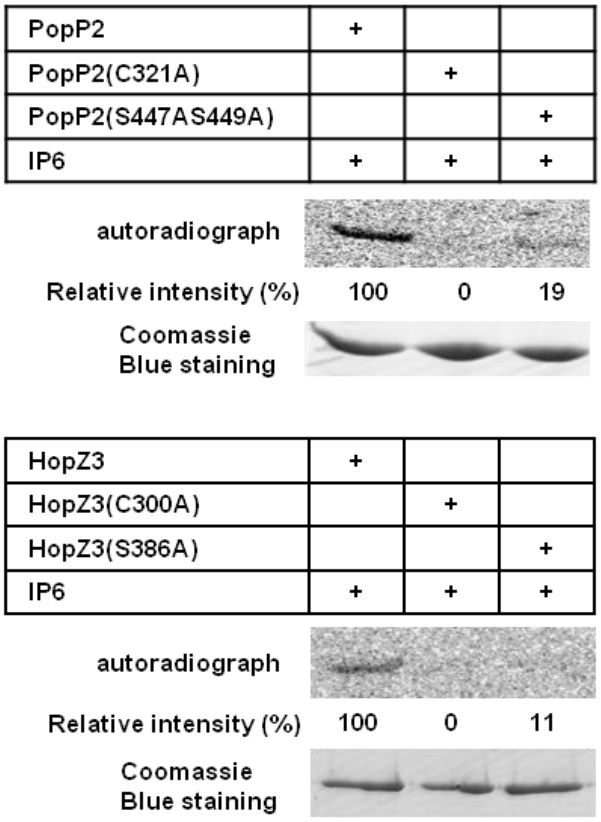


Figure 1.20. The conserved serine residues are also required for the acetyltransferase activity of other YopJ family effectors.

Purified tag-free PopP2, the catalytic mutant PopP2(C321A), and PopP2(S447AS449A) , His tagged HopZ3, the catalytic mutant HopZ3(C300A), and HopZ3(S386A) were subjected to *in vitro* acetylation assay. Numbers underneath the autoradiograph indicates relative acetylation levels of PopP2 mutants (upper panel) or HopZ3 mutants (lower panel) compared to wild-type protein (as 100%). Equal loading of the proteins was confirmed by Coomassie blue staining.

These experiments were repeated three times with similar results.



A central linker region is important for HopZ1a function

The binding of IP6 to HopZ1a and the subsequent conformational change suggested that HopZ1a structure could be stabilized by a cofactor. My collaborator Dr. Zhimin Zhang analyzed the secondary structure of HopZ1a and predicted a central flexible linker region between the central catalytic triad and the conserved C-terminal region containing K289, S349 and S351 that may be important for HopZ1a conformational change. This prediction prompted me to test the requirement of different regions for HopZ1a function. Similar truncates of the highly similar allele HopZ1b was also tested.

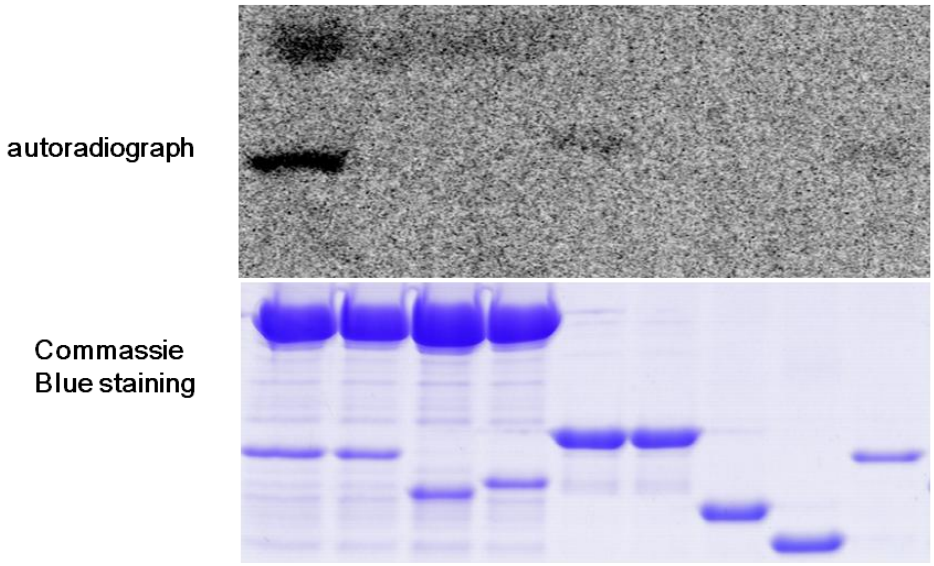
I showed that the central linker region is required for HopZ1a autoacetylation while the N-terminal region containing the first 31 amino acids was dispensable. However no acetylation could be detected when the truncation was made up to 66 amino acids. Interestingly, by using a higher input of HopZ1b, weak autoacetylation activity could also be detected from HopZ1b; the signal was higher than the catalytic mutant HopZ1b (C212A), indicating that the weak acetylation was not a background artifact. The corresponding linker region of HopZ1b was not required for the weak HopZ1b acetylation activity (Figure 1.21).

Figure 1.21. HopZ1b has weaker acetyltransferase activity *in vitro* and a central linker region is important for HopZ1a function.

1 µg HopZ1a truncated proteins and 3µg HopZ1b truncated proteins were subjected to *in vitro* acetylation assay. MBP-JAZ6 was added as a substrate for *trans*-acetylation activity of HopZ1a. IP6 was supplemented as a cofactor. Equal loading of the proteins was confirmed by Coomassie blue staining.

The experiment was repeated twice with consistent results.

Z1a (32-369)	+								
Z1a(C216A) (32-369)		+							
Z1a (67-369)			+						
Z1a (Δ245-281)				+					
Z1b (44-368)					+				
Z1b (C212A) (44-368)						+			
Z1b (44-303)							+		
Z1b (44-262)								+	
Z1b (Δ241-270)									+
MBP-AtJAZ6	+	+	+	+					



DISCUSSION

Several YopJ family effectors are shown to be acetyltransferases, including HopZ1a. Based on the proposed "ping-pong" model, autoacetylation of YopJ family effectors could serve as an acetyl-enzyme intermediate (Mukherjee et al., 2006) for the subsequent transfer of the acetyl group to substrates. K289 was previously hypothesized to be the autoacetylation site of HopZ1a (Lee et al., 2012). However, K289 was not identified as being acetylated in our mass spectrometry analysis. Importantly, *in vitro* assays showed that autoacetylation of HopZ1a was not weakened by the K289R mutation. This is inconsistent with the complete loss of autoacetylation observed in HopZ1a(K289R) from a previous study (Lee et al., 2012). This discrepancy could be explained by the different constructs used in this study (i.e. tag-free proteins) and the previous publication (GST-tagged proteins). Nonetheless, a significant decrease in *trans* acetylation of AtJAZ6 by HopZ1a(K289R) was still observed from an *in vitro* acetylation study carried out by a previous Ph.D student, Dr. Shushu Jiang, in the lab, suggesting that K289 still plays an important role in the biological function of HopZ1a, but not likely by serving as the autoacetylation site. Interestingly, HopZ1a(K289R) mutant failed to induce conformational change upon addition of IP6, indicating that K289 is more likely involved in cofactor interaction. In particular, the replacement of a lysine with arginine may not fully represent the role of K289 because the positively charged arginine may still interact with the negatively charged IP6, even though a significant change in conformation was not observed. In the future, a lysine to glutamic acid mutation should be considered to clarify the function of K289. During the preparation of this dissertation, a study on

HopZ1a(K289R) was published (Rufian et al., 2015), and showed that HopZ1a(K289R) is not completely abolished in biological function. This finding is consistent to my observation that HopZ1a(K289R) still elicits cell death in *N. benthamiana* and partially lost its *trans*-acetylation activity.

A similar partial loss of function was also observed in another YopJ family effector, AvrBsT, where the corresponding lysine residue K282 is required for *trans* acetylation, but not for autoacetylation (Cheong et al., 2014). Interestingly, I constantly observed enhanced autoacetylation signals from HopZ1a(K289R) compared to wild-type HopZ1a. It is possible that other residues that are normally not acetylated in HopZ1a could now be acetylated upon the mutation on K289; or the autoacetylation site is locked in the acetyl-enzyme intermediate form due to suppression of *trans* acetylation caused by the K289R substitution.

Using mass spectrometry, T346 was the only autoacetylation site identified from HopZ1a. This experiment was repeated twice in two mass spectrometry facilities with identical results. However, autoacetylation of HopZ1a was not weakened upon mutation of T346. This result suggests that other residue(s) could serve as supplementary acetylation site(s) when T346 is absent and this hypothesis is also confirmed by another mass spectrometry analysis on HopZ1a(T346A). Indeed, T346 is not conserved in YopJ family effectors. Nonetheless, mutations on two neighboring residues S349 and S351 significantly affect the enzymatic activity of HopZ1a. Even though these two serine residues may not supplement T346 as the alternative autoacetylation site(s) in the mutant HopZ1a(T346A), these residues are required for HopZ1a function.

I also demonstrated for the first time that HopZ1a could inhibit stomatal defense. Interestingly, another T3SE AvrB was recently shown to induce JAZs degradation and reopens stomata through targeting of RIN4 and AHA1 (Zhou et al., 2015b). It will be interesting to know whether inhibition of stomatal closure by HopZ1a is also correlated with degradation of multiple JAZs.

The acetyltransferase activity of several YopJ family effectors can be activated by IP6 as the co-factor (Mittal et al., 2010; Lee et al., 2012; Cheong et al., 2014). As a natural product that is present in a relatively high abundance in eukaryotes, the absence of IP6 in bacteria suggests that the enzymatic activity of YopJ family effectors is enhanced after they are delivered into the hosts (Mittal et al., 2010). IP6 induces conformational changes of AvrA (Mittal et al., 2010). Using NMR, I also observed the transition of HopZ1a to a more compact state in the presence of IP6. Importantly, my data demonstrated that K289, S349 and S351 are required for HopZ1a association with IP6 and/or IP6-induced conformation changes. Consistent with a role in IP6-induced conformational changes, the enhancement of HopZ1a activity by IP6 was lost in HopZ1a(S349AS351A). Furthermore, the association of HopZ1a with IP6 is not dependent on the catalytic residue C216, suggesting that the function of these residues in the C-terminal domain are distinctive from the catalytic residues, which are in the central region of the proteins.

Small molecule-induced allosteric regulation is a common regulatory mechanism of enzymatic activities. IP6 is implicated as an important component in the regulation of various proteins. For instances, IP6 induces a conformational change of the normally

disordered RNA editing enzyme hADAR2 (Macbeth et al., 2005), a vesicle membrane protein synaptotagmin I (Joung et al., 2012), and a sensory perception-related protein arrestin (Zhuang et al., 2010). IP6 is also known to activate bacterial toxins including repeats in toxin (RTX) produced by *Vibrio cholera* (Lupardus et al., 2008) and toxin A produced by *Clostridium difficile* (Pruitt et al., 2009) after they enter the animal cells. The known IP6 binding motifs often include a basic pocket with polar residues, such as lysine. Serine residues are also involved in the interaction through stabilization of protein-IP6 interaction by hydrogen bonds. Therefore, it is likely that the residues under study are part of the IP6-binding pocket in YopJ family effectors, and the resultant conformation changes enhance their enzymatic activities.

In order to understand more details about IP6-induced changes in protein conformation, HopZ1a mutant with a deletion of a central linker region was tested and this experiment showed that the "linker" region was required for HopZ1a autoacetylation activity. As the deletion of the whole linker region likely disrupted the overall structure of HopZ1a, substitution with amino acid with low flexibility, such as proline coupled, with conformation characterization using NMR will provide more insight into the role of this region in HopZ1a activation process.

The conservation of serine residues among YopJ effectors and their requirement for the enzymatic activities of PopP2 and HopZ3 suggest that the serine residues serve an essential role in the activity of this family of acetyltransferases. The presence of a conserved C-terminal domain has been proposed previously (Mukherjee et al., 2007). My analyses suggest that the conserved serine residues in the C-terminal domain contribute to

the activation of acetyltransferase activity after the effectors enter eukaryotic hosts via co-factor interaction. Since autoacetylation was almost completely lost in PopP2(S447AS449A) and HopZ3(386A), it is possible that the conserved serines in PopP2 and HopZ3 also serve as autoacetylation sites.

In summary, I reported two conserved serine residues in the C-terminal domain of HopZ1a that are required for the biological function of HopZ1a as evidenced by the loss of HR-triggering ability, and suppression of defense responses including callose deposition and stomatal closure. I also showed that these serine residues are important for the enzymatic activity of HopZ1a and are indispensable for IP6-induced activation in eukaryotic hosts. Even though K289 is not directly required for autoacetylation, it seems to be involved in HopZ1a-IP6 interaction. This work provides insights about the enzymatic activity regulation of an important family of bacterial effectors in the hosts. As bacterial effectors are potent virulence factors during infection, the knowledge may potentially be applied to the identification of chemical inhibitors of YopJ family effectors.

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Chapter II. Functional study of the HopZ1a-Interacting protein, GmZINP2, in soybean defense

ABSTRACT

Gram negative bacteria use a Type III secretion system (T3SS) to secrete different effectors into the plant host to suppress immunity. HopZ1a is one of the effectors secreted by *Pseudomonas syringae*. In Arabidopsis, HopZ1a targets JAZ proteins to promote bacterial infection. In soybean, HopZ1a induces the degradation of an isoflavone biosynthetic enzyme GmHID1 for virulence. Here, I report the identification of another HopZ1a target in soybean named as GmZINP2. GmZINP2 negatively regulates soybean defense against *P. syringae* isolate *Pgy*BR1-O1. GmZINP2 is a modular protein, carrying an N-terminal DEK-C domain and a C-terminal SWIB domain. These two domains are both implicated with functions associated with the chromatin. I showed that GmZINP2 binds to DNA and interacts with a MYB class transcription factor GmMYB56. HopZ1a acetylates GmZINP2 and affects the interaction between GmZINP2 and GmMYB56. This study provides an example of how T3SE can potentially suppress defense through modulation of chromatin-associated process.

INTRODUCTION

Due to their sessile nature, plants have to cope with a multitude of microbes that potentially harm the host as pathogens. Even though perception of evolutionary conserved molecular pattern and activation of PAMP-triggered immunity (PTI) is efficient in deterring most pathogen infection, Gram negative bacteria use Type III secretion system (T3SS) to defeat PTI response through secretion of PTI suppressing Type III secreted effectors (T3SEs) (Chisholm et al., 2006; Spoel and Dong, 2012). The identification of T3SE targets and possible manipulation of them to suppress defense provide much information about the plant innate immunity system (Boller and He, 2009; Block and Alfano, 2011; Deslandes and Rivas, 2012; Feng and Zhou, 2012). In the species *Pseudomonas syringae*, more than 80 T3SEs are being identified among different pathovars (Lindeberg et al., 2006). Despite tens of T3SE are being confirmed, the discovery of new effectors targets still never stop to surprise us about the remarkably diverse approaches a pathogen may take for virulence.

HopZ1a is a Type III secreted effector (T3SE) from *Psy*A2 that belongs to the YopJ family of acetyltransferases (Ma et al., 2006). In Arabidopsis, HopZ1a acetylates the key regulators of the JA signaling, JAZs, and triggers their degradation. Degradation of JAZs lead to derepression of JA signaling and thereby suppresses defense against *Pto* through the SA pathways (Jiang et al., 2013). HopZ1a also acetylates tubulin and affect antimicrobial secretory pathway, possibly through the disruption of microtubule network (Lee et al., 2012). During incompatible interaction, HopZ1a also acetylates a non-

function pseudokinase, ZED1, which is associated with a CC-NB-LRR protein ZAR1 to trigger HopZ1a-specific defense (Lewis et al., 2010; Lewis et al., 2013).

So far, only one target of HopZ1a has been reported in soybean. HopZ1a targets an isoflavone biosynthetic gene, GmHID1, for degradation with an unknown mechanism. The catalytic residue of HopZ1a is required to trigger GmHID1 degradation, suggesting that an enzymatic activity dependent process is involved. GmHID1 degradation is associated with a decrease of the precursor of phytoalexin, which is produced as antimicrobial metabolites to inhibit infection (Akashi et al., 2005; Lozovaya et al., 2007; Bednarek and Osbourn, 2009; Zhou et al., 2011). In order to understand more about the cellular functions of HopZ1a in soybean, a natural host of *P. syringae*, yeast-two-hybrid screening for HopZ1a-associating proteins was carried out by a previous postdoc in the lab, Dr. Huanbin Zhou. From this screen, a protein with potential chromatin remodeling function, GmZINP2, was identified. Chromatin is implicated in diverse functions from growth, development to response to environmental signals through global transcription reprogramming in plants (Ho and Crabtree, 2010; Li and Reinberg, 2011; van Zanten et al., 2012). However, our knowledge on how chromatin may regulate defense responses is limited (Burg and Takken, 2009; Alvarez et al., 2010; Ma et al., 2011; Berr et al., 2012) and thus GmZINP2 is selected for further characterization.

Eukaryotic DNA is wrapped around an octamer of core histone proteins in a "bead-on-a-string" model to form the basic structural unit called nucleasome. Nucleasomes are further condensed in the form of 30nm fiber. The accessibility of DNA is regulated through different means. DNA can be directly methylated during gene

silencing processes (Schubeler, 2015). Alternatively, the tails of core histone can be modified by acetylation, methylation, phosphorylation, sumoylation, ubiquitination, glycosylation and ADP-ribosylation. The combination of these modifications at different positions significantly affects the outcome of transcription. (Fuchs et al., 2006; Berger, 2007; Bannister and Kouzarides, 2011; Hottiger, 2011). Finally, a group of ATP driven chromatin remodeling proteins can directly alter the configuration of chromatin by sliding, displacement, remodeling and replacement mechanisms (Jerzmanowski, 2007; Clapier and Cairns, 2009; Hargreaves and Crabtree, 2011; Gentry and Hennig, 2014). Together these mechanisms either activate or repress gene expression at a global level.

In plants, several ATP-dependent chromatin-remodeling proteins have been demonstrated to regulate plant defense responses. For examples, SYD and BRM both regulate expression of defense related genes including *PR1* (Bezhani et al., 2007; Walley et al., 2008). SYD is recruited to the promoters of these genes, possibly affecting their transcription through chromatin remodeling (Walley et al., 2008). BRM may be guided to the promoters of different target genes including positive regulator of gibberellins signaling, through the bromodomain and several DNA binding regions (Farrona et al., 2007; Archacki et al., 2013). Alternatively, chromatin remodeling protein including SWI3C, brings the GA suppressor DELLA and its modifier SPY together, indicating a possible modification mechanism involved independent of the chromatin level (Archacki et al., 2013).PIE1 negatively regulates defense against *Pto*DC3000 by depositing the histone variant H2A.Z to PIE1-regulated loci. It is possible that defense related genes are repressed by PIE1-mediated deposition of H2A.Z in the absence of pathogen infection

(Sarnowska et al., 2013). Notably, another PIE associated protein, ARP6, is also involved to regulate defense response in a temperature-dependent manner (Deal et al., 2007; March-Diaz et al., 2008). DDM1 is another chromatin remodeling protein that regulates DNA methylation by a still unknown mechanism (Cheng et al., 2013).

GmZINP2 has a modular structure with an N-terminal DEK-C domain and a C-terminal SWIB domain. The DEK-C domain is a DNA binding domain of DEK; and mis-regulation of DEK-C domain containing proteins are associated with cancer and autoimmune disease in human (Riveiro-Falkenbach and Soengas, 2010; Vinnedge et al., 2013) and deficiency in stress response in plants (Waidmann et al., 2014a). The SWIB domain is a protein-protein interaction domain with chromatin remodeling function (Bennett-Lovsey et al., 2002; Reisman et al., 2009).

I showed that GmZINP2 is a negative regulator of defense and over-expression of GmZINP2 in soybean suppressed early flg22-mediated ROS burst and later induction of *PR* gene expression. HopZ1a interacts with and acetylates GmZINP2. Even though the DNA binding activity of GmZINP2 was not affected by HopZ1a, I showed that the interaction between GmZINP2 and GmMYB56 was affected by HopZ1a. These data adds to our current list of examples that chromatin plays an important role in plant innate immunity and possibly serve as a virulence target of T3SE.

MATERIALS AND METHODS

Bacterial strains and plasmids

P. syringae pv .*glycinea* BR1-O1 (*Pgy*BR1-O1). *P. syringae* pv. *Tomato* DC3000 (*Pto*DC3000) and *P. syringae* pv. *tomato hrcC* (Roine et al., 1997) were grown at 30℃ in King's B medium (King et al., 1954). *Escherichia coli* strains DH5α and BL21 were grown in Luria–Bertani (LB) medium and 37℃ as described previously (Morgan et al., 2010). *P. syringae* transformation was conducted by electroporation using an Eppendorf electroporator 2510 (Eppendorf North America, Westbury, NY, USA) according to the manufacturer's instructions. List of bacteria strains and plasmids used in this study are summarized in Table 2.1.

Plant materials and growth conditions

Soybean (*Glycine max* (L.) cv. Williams 82) seeds were surface sterilized with 10% bleach for 10 min and pre-germinated on wet filter paper at room temperature in the dark for 4 days. Seedlings were transplanted to soil and grown in a conditioned growth room (19-21 ℃, 16-hour photoperiod, and relative humidity of 75-80%).

Full length *GmZINP2* carrying N-terminal Flag tag was cloned into pAVA319. The whole segment spanning the 2X 35S promoter, full length GmZINP2 and the terminator sequence from pAVA319 was subcloned into the vector pTF101.1. The resultant construct was confirmed by sequencing and restriction enzyme digestion before sending to plant transformation facility at the Iowa State University for generation of transgenic soybean. T1 generation of transgenic soybean was germinated as described above and

then screened by spraying with herbicide BASTA. Over-expression of *GmZINP2* was confirmed by quantitative RT-PCR (qRT-PCR) and western blotting using anti-Flag antibody.

Protein expression and purification

Wild-type and mutant *HopZ1a, HsDEK(270-375)* were cloned into plasmid vector pRSFDuet-1 (Novagen, Madison, WI) containing a 6×His-SUMO tag, and transformed in *E. coli* strain BL21 (DE3). Full length *GmZINP2*, *GmZINP2(1-144)* and *GmZINP2(145-333)*, *GmMYB56* were cloned into pET28a. Except for full length GmZINP2, which was induced at OD_{600} = 0.6 (approximately 6×10^8 cfu/mL) with a final concentration of 0.1mM IPTG at 16°C overnight, all other proteins were induced at OD_{600} = 0.6 (approximately 6×10^8 cfu/mL) with a final concentration of 1mM IPTG at 22°C overnight. Recombinant 6×His-SUMO-tagged proteins were purified using nickel resin, and the 6×His-SUMO tag was subsequently removed by ULP1 protease as described previously (Jiang et al., 2013).

Yeast-two-hybrid assays

Full length *HopZ1a* or *GmMYB56* were cloned into the bait vector in pGBKT7 and full length *GmZINP2* or *HopZ1a* were cloned into the prey vector in pGADT7. Recombinant plasmids were co-transformed into yeast strain AH109. The empty vectors (EV) were also transformed as negative controls. Transformants carrying both plasmids were selected on a synthetic medium supplemented with dropout solution (SD-2) plates. Individual colonies were subsequently transferred to SD-4 medium to examine protein-protein interactions between different combinations.

In vitro acetylation assays

An *in vitro* acetylation assay was used to examine the acetyltransferase activity of HopZ1a and different mutants or *trans*-acetylation activity on MBP-JAZ6, GmZINP2, GmZINP2(1-144) and GmZINP(145-333). To determine the autoacetylation level, 1 µg HopZ1a or mutants, was incubated with 1 µL [14C]-acetyl-CoA (55 µci/µmol) in 25 µL of reaction buffer (50 mM HEPES (pH 8.0), 10% glycerol, 1 mM DTT, 1 mM PMSF, 10 mM sodium butyrate) at 30 ℃ for one hour. 100 nM IP6 was supplemented to the reaction when appropriate. To determine the *trans* acetylation, 7 µg MBP-AtJAZ6-HIS, GmZINP2, GmZINP2(1-144) or GmZINP2(145-333) was used in each reaction as the substrate. The reactions were stopped by addition of 2x Laemmli buffer and then subjected to SDS-PAGE. Acetylated proteins were detected by autoradiography as previously described (Jiang et al., 2013). After autoradiography, the protein gels were removed from the filter paper and stained with Coomassie Blue as a loading control.

Pseudomonas syringae infection assays

To test the HR-triggering activity of HopZ1a and bacterial multiplication of pgy BR1-O1 on wild type soybean and transgenic soybean overexpressing GmZINP2, fully expanded primary leaves of 14-day old soybean (cultivar Williams 82) were infiltrated with bacterial suspensions at an OD_{600} = 0.1 (approximately 1×10^8 cfu/mL) for HR-triggering experiment and OD_{600} = 0.005 (approximately 5×10^6 cfu/mL) for bacterial multiplication assay for *Pgy*BR1-O1. Cell death symptoms were observed 36 hpi for *Pgy*BR1-O1 after clearance with 95% ethanol. The bacterial populations were determined

as colony forming units (cfu) per cm^2 four days after inoculation using a previously described procedure (Morgan et al., 2010).

ROS burst assay

1cm^2 leaf disc was taken from the cotyledons of 14-day old soybean plants (use of older plants significantly affects the result of this assay). The leaf disc was cut by a sharp razor blade into four equal pieces. Small leaf pieces were then transferred to 96-well plates with water and rest overnight with continuous illumination. Before the experiment, water was carefully removed and then 200 µL reaction mixture containing 12.5 µg/µL peroxidase from horseradish (hrp) (Sigma-Aldrich, St. Louis, MO), 88.5 µg/µL freshly prepared luminol (Sigma-Aldrich, St. Louis, MO) were added. 5 µM flg22 (PhytoTechnology Laboratories, Shawnee Mission, KS) was supplemented as an elicitor when necessary. The plate is then transferred to a microplate reader (Tecan Infinite 200 Pro) and the relative luminescence was measured with an integration time of 500ms, 15s per cycle for a total of 60 minutes.

Quantitative RT-PCR

14-day old soybean plants were infiltrated with OD_{600} = 0.2 (approximately 2×10^8 cfu/mL) *Pgy*BR1-O1. Samples were taken at 48 hpi and total RNA was extracted. 2 µg DNase-treated total RNA was reverse transcribed by Revert Aid reverse transcriptase at 42 ℃ for one hour using Oligo-dT as primer. Diluted cDNA was then subjected to qPCR analysis using iQ SYBR Green supermix (BioRad) and Bio-Rad CFX96. The relative expression levels were determined using the internal control *GmUbi* and calculated by the $2^{-\Delta\Delta ct}$ method.

GmPR1 F: 5'-AACTATGCTCCCCCTGGCAACTATATTG-3'

GmPR1 R: 5'-TCTGAAGTGGTAGCTTCTACATCGAAACAA-3'

GmZINP2 F: 5'-GACGAGATCAACTTCCAGCA-3'

GmZINP2 R: 5'-CACTTTCTTTGGGCACTTGA-3'

GmUbi F: 5'-TGTGTAATGTTGGATGTGTTCCC-3'

GmUbi R: 5'-ACACAATTGAGTTCAACACAAACC-3'

Electromobility gel shift assay (EMSA)

EMSA was carried out as described previously with minor modifications (Waldmann et al., 2003). 0.1 ρmol Litmus 28i plasmid was incubated with increasing amount of 6XHis-SUMO-DEK(270-375), GmZINP2, GmZINP2(1-144) or GmZINP2(145-333) in 20 μL reaction buffer (20 mM HEPES–KOH, pH=7.6, 100 mM NaCl, 1 mM EDTA, 1 mM1mM DTT) at 37°C for 1 hour. 6× loading dye was added and nucleoprotein complexes were separated on 0.6% agarose gels in 0.5× TBE (90 mM Tris, 89 mM boric acid, 2 mM EDTA pH=8) for 16 hours at 2 V/cm inside a cold room. To study the effect of acetylation on DNA binding, GmZINP2(145-333) was acetylated by HopZ1a using the procedure described in the section of "*in vitro* acetylation assays" in a 10 μL reaction volume using C12 acetyl-CoA without glycerol, followed by incubation with Litmus 28i as described above. C14 acetyl-CoA was used instead of C12 acetyl-CoA for visualization of acetylation by radiography.

***In vitro* GST-pull down assay**

GST pull-down was carried out as described (Jiang et al., 2013). Briefly, GST-GmZINP2(145-333) was incubated with 30 μL glutathione agarose beads (Invitrogen) for

one hour at 4°C. The beads were washed with TKET150 (20 mM Tris-HCl (pH=7.5), 150 mM KCl, 0.1 mM EDTA and 0.05% Triton X-100) five times and then incubated with purified GmMYB56 proteins at 4°C overnight. Various amount of tag free HopZ1a or HopZ1a(C216A) were also added. The beads were washed for three times and the presence of the GmMYB56 proteins on the beads was detected by western blotting using anti-His antibodies conjugated with horseradish peroxidase (HRP) (Santa Cruz Biotechnology Inc.).

Table 2.1. Bacterial strains and plasmids used in Chapter II.

Strains or Plasmids	Description	Source/ reference
Escherichia coli DH5α	F- Φ80d*lac*ZΔM15 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 hsdS$_B$(r$_B$⁻ m$_B$⁻)* λ(DE3 [*lacI lacUV5-T7 gene 1 ind1 sam7 nin5*]	Invitrogen
Pseudomonas syringae pv. *tomato* strain DC3000	Isolated from tomato plants, also infects *Arabidopsis*, Rif^R	Cuppels, 1986
Pseudomonas syringae pv. *tomato* strain *hrcC*	Isolated from tomato plants, also infects *Arabidopsis*, Rif^R	Roine *et al.*, 1997
Pseudomonas syringae pv. *glycinea* BR1Rif-O1	A mutant of *Pgy*BR1, Rifampicin spontaneous mutant with the endogenous plasmid carrying HopZ1b cured	Zhou *et al* ., 2009
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
pGEX4T-2::*HopZ1a*	pGEX4T-2 carrying *hopZ1a*, Amp^R	Zhou *et al.*, 2011
pGEX4T-2::*HopZ1a(C216A)*	pGEX4T-2 carrying *hopZ1a(C216A)*, Amp^R	Zhou *et al.*, 2011
pGEX4T-2::*GmZINP2(145-333)*	pGEX4T-2 carrying *GmZINP2(145-333)*, EcoR1/Sal1,Amp^R	this study
pET-mal::*AtJAZ6*	pET-mal carrying *AtJAZ* genes, Kan^R	Jiang *et al.*, 2013
pRSF::*HopZ1a*	N-terminal 6×His-SUMO tagged *HopZ1a* . Kan^R	Jiang *et al.*, 2013
pRSF::*HopZ1a(C216A)*	N-terminal 6×His-SUMO tagged *HopZ1a(C216A)*. Kan^R	Jiang *et al.*, 2013
pRSF::*DEK(270-375)*	N-terminal 6×His-SUMO tagged *DEK* with deletion of N-terminal 269 aa. BamH1/Xhol1. Kan^R	this study
pET28a::*GmZINP2*	N-terminal 6×His tagged *GmZINP2*. Nco1/Xhol1,Kan^R	Huanbin Zhou

pET28a::*GmZINP2(1-144)*	N-terminal 6×His tagged *GmZINP2*. Nco1, Xhol1 Kan^R	this study
pET28a::*GmZINP2(145-333)*	N-terminal 6×His tagged *GmZINP2*. Nco1,Xhol1Kan^R	this study
pET28a::*GmMYB56*	N-terminal 6×His tagged *GmMYB56*. Kan^R	Huanbin Zhou
pGBKT7::Hop*Z1a*	Yeast BD vector carrying N-terminus c-myc tagged *HopZ1a*. Kan^R	Huanbin Zhou
pGBKT7::*GmMYB56*	Yeast BD vector carrying N-terminus c-myc tagged *GmMYB56*. Kan^R	this study
pGAD7::Hop*Z1a*	Yeast AD vector carrying N-terminus HA tagged *HopZ1a*. Amp^R	Huanbin Zhou
pGAD7::*GmZINP2*	Yeast AD vector carrying N-terminus HA tagged *GmZINP2*. Amp^R	Huanbin Zhou
pTF101.1	Binary vector for Agrobacterium mediated transformation of soybean. $Spec^R$	PTF Iowas State University
pTF101.1::*GmSWI7*	pTF101.1 carrying 2X 35S promoter, N-terminus Flag tagged *GmZINP2* and terminator sequence as a Sma1 blunt end insertion. Spe^R	this study
pEG100::*3XFlag-at4g22360*	pEG100 carrying *at4g22360* tagged with 3XFlag at N-terminus,EcoR1/Sac1. Kan^R	Jiang *et al.*, 2013

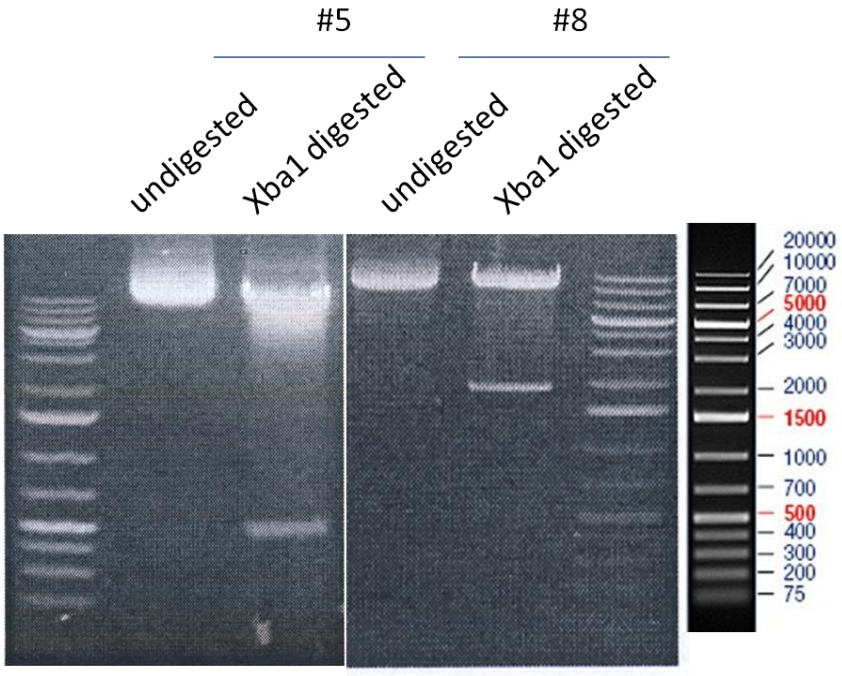
RESULTS

Generation of transgenic soybean overexpressing GmZINP2

In order to study the function of GmZINP2 in the native system i.e. soybean, I prepared the constructs over-expressing *Flag-GmZINP2* under the constitutive 35S promoter in the pTF101.1 vector (from Iowa State University, plant transformation facility). The constructs were confirmed by sequencing and restriction digestion (Figure 2.1). The constructs were named as pTF:pava-GMSW17#5 and pTF:pava-GMSWI7#8. The two constructs were sent to the plant transformation facility (PTF) at the Iowa State University. There, these constructs were introduced into *Agrobacterium tumefaciens* strain EHA101 and used to transform the soybean cultivar Williams 82.

Figure 2.1. Confirmation of *GmZINP2* cloning into the vector pTF101.1.

Full length *GmZINP2* with an N-terminal Flag tag under the control of the constitutive *35S* promoter and a terminator sequence (2.7kb) was blunt-end ligated into *Sma*I digested pTF101.1 vector. The inserted sequence contains a unique *Xba*I site. Depending on the orientation of the insertion, *Xba*I digestion produced a band of 500 bp for construct #5 and 2100 bp for construct #8.



GmZINP2 is a negative regulator of defense in soybean

The seeds from the transformed soybean were germinated and the transgenic seedlings were selected by BASTA. Out of the six independent transformation events, only line 184.12 showed increased expression of *GmZINP2* at both the RNA level and protein level (Figure 2.2). Therefore, line 184.12 was used for subsequent pathological studies while the remaining five lines were used as negative controls.

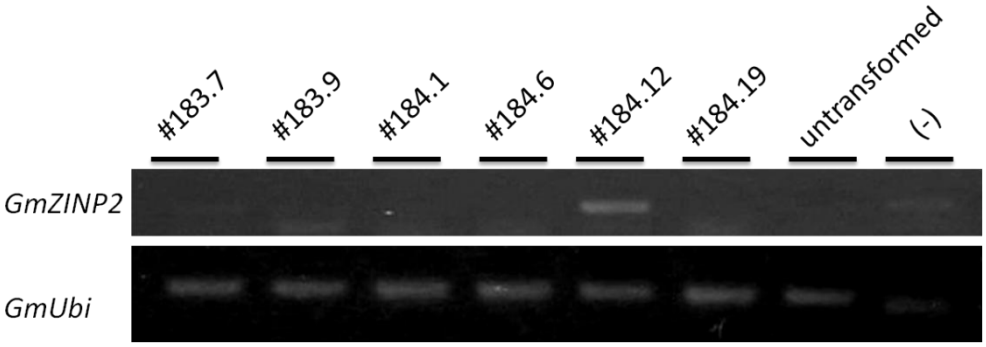
First, I tested the role of GmZINP2 in defense against *P. syringae* pv. *glycinea* BR1-O1. About 10 fold increase in bacterial multiplication was observed in *GmZINP2*-overexpressing line at 4 dpi after the plants were inoculated with *Pgy*BR1-O1. Such an increase was observed in both *Pgy*BR1-O1 carrying an empty vector (EV) or expressing HopZ1a (Figure 2.3), suggesting that GmZINP2 is a general negative regulator of defense involved in both PTI and ETI responses.

Figure 2.2. Confirmation of transgenic soybean over-expressing *35S-GmZINP2*.

A, Total RNA was extracted from transgenic soybean from independent transformation events. DNase treated sample was reverse transcribed and diluted cDNA was used for PCR amplification using gene specific primers for *GmZINP2*. *GmUbi* was used as an internal control.

B, Expression of Flag-GmZINP2 was confirmed by western blotting using anti-Flag antibody (Santa cruz Biotechnology).

A



B

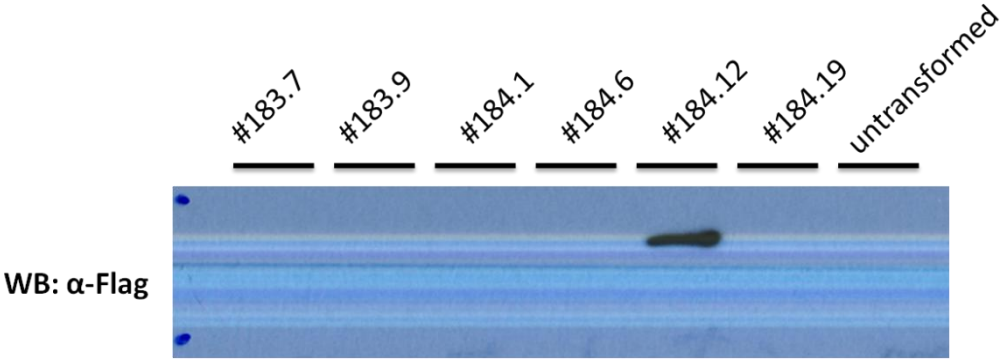
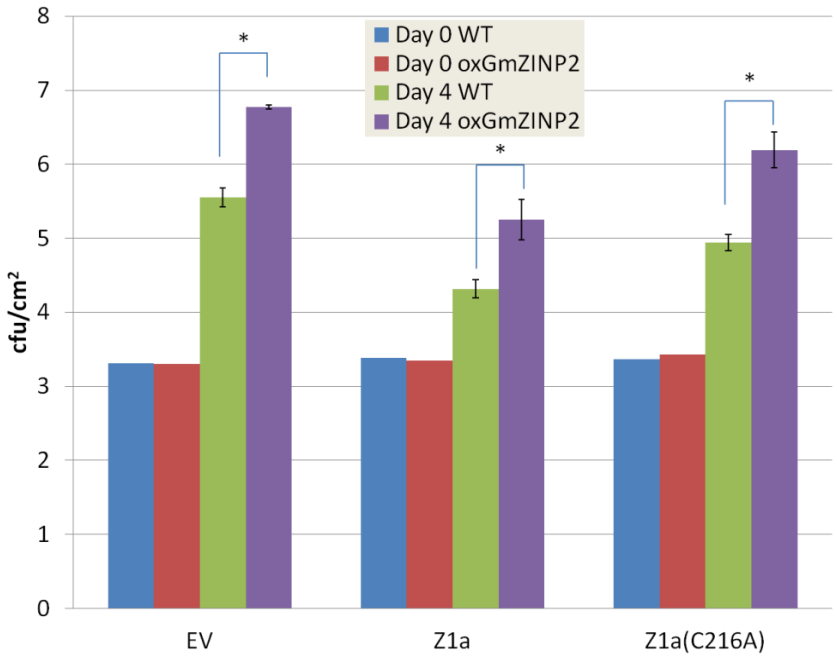


Figure 2.3. GmZINP2 is a negative regulator of defense against *P. syringae*.

14-day old transgenic soybean over-expressing *GmZINP2* (#184.12) and control plant (#184.19) were infiltrated with *Pgy*BR1-O1 carrying pUCP20tk (EV), or expressing wild-type or mutant HopZ1a at OD_{600} = 0.005 ($5x10^6$ cfu/mL). Bacterial populations were determined at 0 and 4 days post inoculation (dpi). Average colony forming units per square centimeter (cfu/cm^2) and standard deviations (as error bars) are presented. Asterisks on top of the bars represent data with statistically significant differences (two tailed t-test $p<0.05$).

The experiment was repeated three times with similar results.



GmZINP2 regulates both early and late defense response upon pathogen perception

As over-expression of GmZINP2 promoted growth of *Pgy*BR1-O1 in soybean, and such an effect was observed in both PTI and ETI response, I was interested to determine at which stage GmZINP2 participates in defense. I used two different approaches to further elaborate the negative roles of GmZINP2 in defense. First, I tested the induction of reactive oxygen species (ROS) burst induced by flg22. As ROS bust happens minutes after flg22 treatment, it is used as an output of early defense response. My experimental results showed that the induction of ROS was attenuated in the GmZINP2-overexpressing line compared to the control (Figure 2.4). Next, I examined the induction of the SA-signaling defense marker gene *GmPR-1* upon *Pgy*BR1-O1 infection. Usually, *GmPR-1* expressed was significantly induced from 24-48 hours post infection and it is used as an output of late defense response. Consistently, the induction of *GmPR-1* was also lowered in the GmZINP2-overexpressing soybean (Figure 2.5).

Figure 2.4. ROS induction is attenuated in soybean overexpressing GmZINP2.

Leaf disc was taken from the cotyledons of 14 day-old soybean plants and allowed to rest overnight in water under continuous illumination. 5 μM flg22 was added to induce ROS burst. The amount of ROS was measured over time by a microplate reader. Average readings from at least three leaf discs and standard error (as error bars) are presented. The experiment was repeated twice with consistent results.

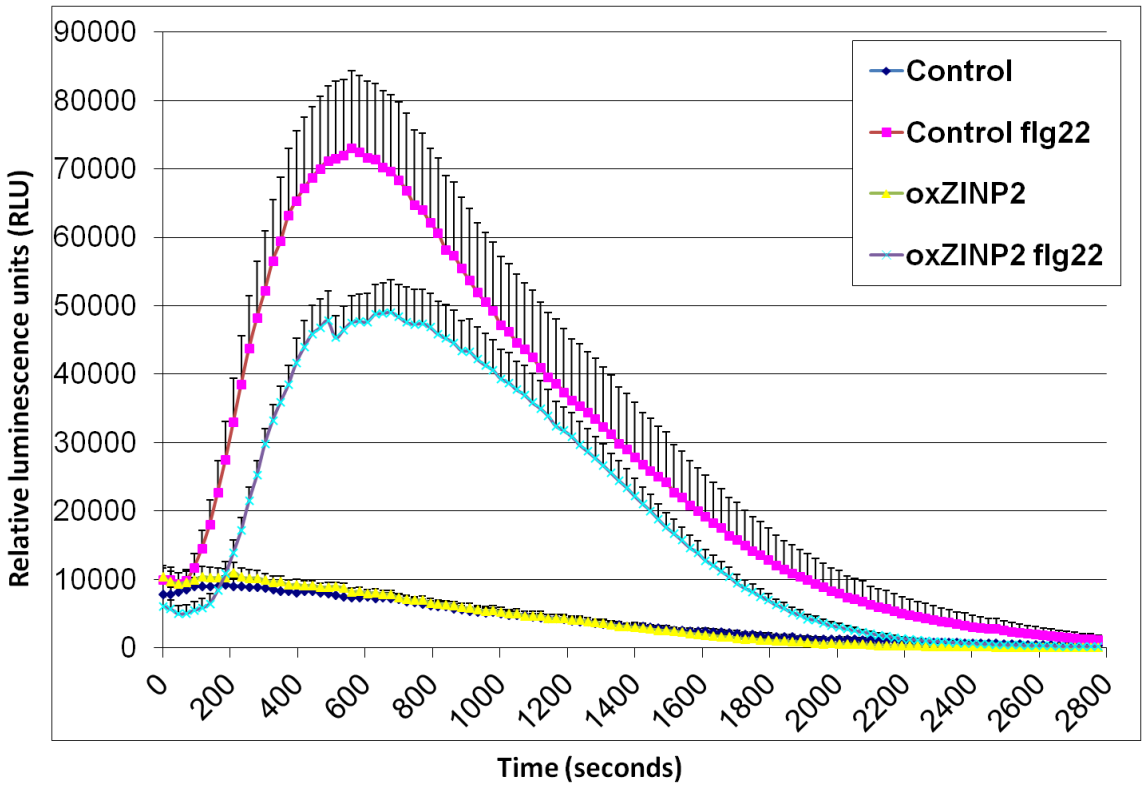
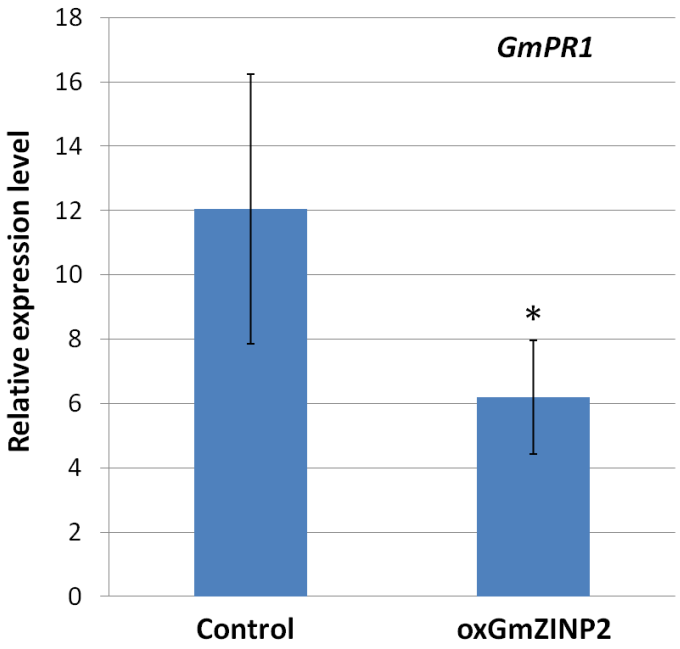


Figure 2.5. *GmPR-1* induction is suppressed in soybean overexpressing GmZINP2.

14-day old soybean seedlings were infiltrated with *Pgy*BR1-O1 at OD_{600} = 0.2 (approximately 2×10^8 cfu/mL). Total RNA was extracted from samples at 48 hpi. DNase-treated total RNA was reverse transcribed and diluted cDNA was used in qPCR analysis using iQ SYBR Green supermix and *GmPR-1* specific primers (Gene Bank accession number BU577813). Relative expression levels of *GmPR1* were calculated compared to control sample plant treated with water only. The expression is normalized by the internal control *GmUbi* and calculated by the $2^{-\Delta\Delta ct}$ method. Standard errors (as error bars) are presented. The asterisk represents data with statistically significant differences (two tailed t-test $p<0.05$).

The experiment was repeated twice with consistent results.



GmZINP2 is a potential chromatin remodeling protein with DNA binding activity

GmZINP2 contains an N-terminal DEK-C domain and a C-terminal SWIB domains. These two domains are associated with proteins with chromatin remodeling function inside the nucleus. As the human DEK protein was known to bind DNA and this activity was important for DEK transcription regulation function (Waldmann et al., 2002; Waldmann et al., 2003), I examined whether GmZINP2 also has a DNA binding activity.

Purified GmZINP2 protein was incubated with the plasmid Litmus 28i to study the general DNA binding properties of GmZINP2 and the migration of DNA after electrophoresis was visualized by ethidium bromide staining. The C-terminal domain of the human DEK protein was used as a positive control. GmZINP2 bound to Litmus 28i and slowed down the migration of Litmus 28i in an electromobility gel shift assay (EMSA). The DNA was trapped inside the loading well when a larger amount of GmZINP2 was added, suggesting that large nucleo-protein complex was formed (Figure 2.6).

Next, I examined which domain is required for GmZINP2 to bind DNA. For this purpose, I constructed two truncated version of GmZINP2: The N-terminal truncate (amino acid 1-144) containing the DEK-C domain and the C-terminal truncate (amino acid 145-333) containing the SWIB domain. The GmZINP2 truncates were used in the same experimental setup as the full-length GmZINP2. Interestingly, the C-terminus, but not the DEK-C containing N-terminus was mainly responsible for the DNA binding activity of GmZINP2 (Figure 2.6).

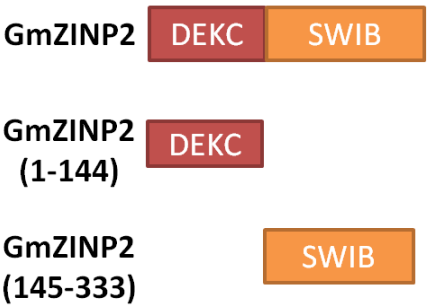
Figure 2.6. The SWIB domain-containing C-terminus of GmZINP2 has DNA binding activity.

A, Schematic representation of two truncates of GmZINP2.

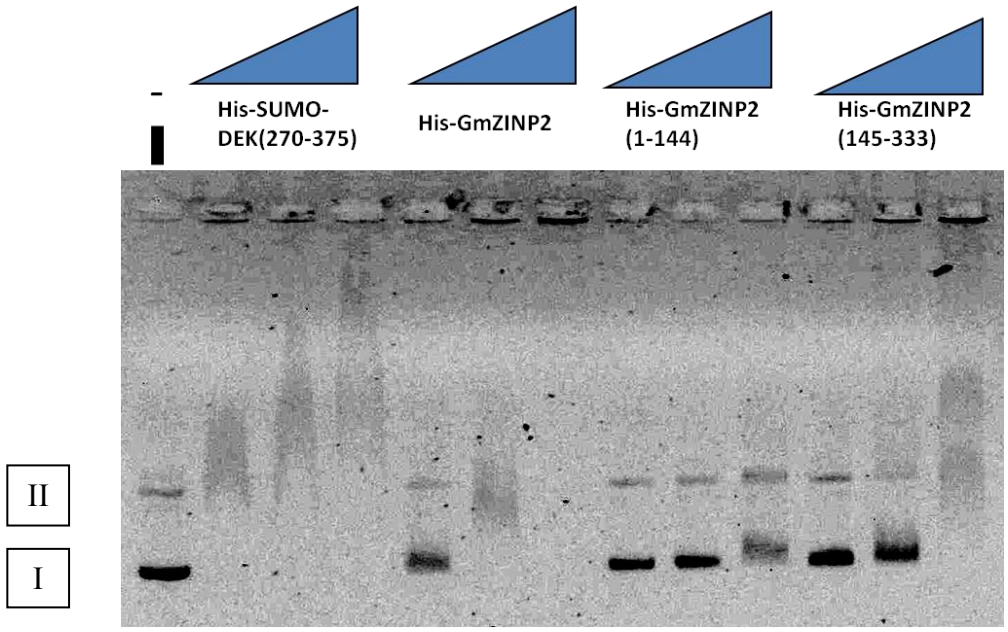
B, 0.1 ρmol Litmus 28i plasmid was incubated with increasing amount of 6XHis-SUMO-DEK(270-375) at molar ratios of DEK/DNA of 30, 90 and 180 (lane 2-4) , full length 6XHis-GmZINP2, GmZINP(1-144) or GmZINP(145-333) at molar ratios of GmZINZP2/DNA of 150, 600 and 1200 (lane 5-12). Nucleoprotein complexes were analyzed on 0.6% agarose gels and visualized by ethidium bromide staining. I and II corresponds to the super-coiled form and linear form of plasmid DNA.

The experiment was repeated twice with consistent results.

A


GmZINP2
DEKC
SWIB
GmZINP2
(1-144)
DEKC
GmZINP2
(145-333)
SWIB

B


-
His-SUMO-
DEK(270-375)
His-GmZINP2
His-GmZINP2
(1-144)
His-GmZINP2
(145-333)
II
I

HopZ1a acetylates GmZINP2

Since the DEK-C domain and SWIB domain may play different roles in plant defense response, I examined which domain was required for GmZINP2 to interact with HopZ1a, which may provide hints on the possible manipulation of GmZINP2 by HopZ1a. GST-tagged HopZ1a or HopZ1a(C216A) were immobilized on glutathione-agarose beads and used to pull down full-length or truncated GmZINP2 protein. GST-only bead was used as a negative control. Both full-length GmZINP2 and the GmZINP2(145-333) containing the SWIB domain were successfully pulled down by HopZ1a. Similar to the interaction between HopZ1a and AtJAZ6, the catalytic mutant HopZ1a(C216A) interacted with GmZINP2 as strongly as wild-type HopZ1a (Figure 2.7).

As HopZ1a was shown to acetylate different proteins in plants, next I examined whether GmZINP2 is an acetylation substrate of HopZ1a. I showed that GmZINP2 could be acetylated by HopZ1a *in vitro*. Interestingly, the C-terminal truncate GmZINP2(145-333) showed a stronger acetylation level than full-length GmZINP2. As expected, the non-interacting N-terminal truncate GmZINP2(1-144) could not be acetylated (Figure 2.8).

Figure 2.7. Interaction between HopZ1a and GmZINP2 requires the C-terminus of GmZINP2.

His tagged GmZINP2, GmZINP2(1-144), GmZINP2(145-333), GST, GST-HopZ1a or GST-HopZ1a(C216A) proteins were expressed in *E. coli*. Pull down of GmZINP2 and GmZINP2(145-333) by HopZ1a was determined by western blotting before (input) and after affinity purification (pull-down) using anti-His antibody (Santa cruz Biotechnology). The protein abundances of GST, GST-HopZ1a and GST-HopZ1a(C216A) on the resins after washes were detected by Coomassie blue staining.

The experiment was repeated once with consistent result.

GST	+				
GST-Z1a		+		+	+
GST-Z1a(C216A)			+		
6XHis-GmZINP2	+	+	+		
6XHis-GmZINP2 (1-144)				+	
6XHis-GmZINP2 (145-333)					+

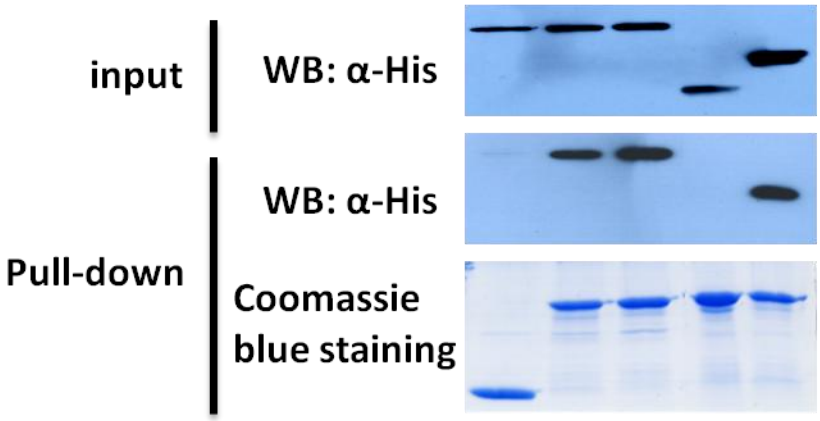
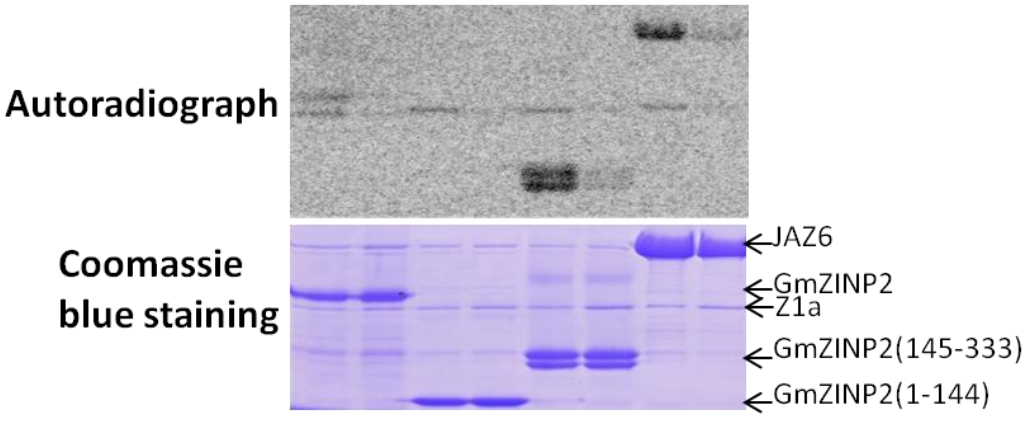


Figure 2.8. GmZINP2 is acetylated by HopZ1a *in vitro*.

His-tagged GmZINP2 and truncates, MBP-AtJAZ6 and tag free HopZ1a(32-369) were subjected to *in vitro* acetylation assay supplemented with 100 nM IP6 as a cofactor. HopZ1a(32-369) was used to distinguish between the similar-sized full length GmZINP2 and HopZ1a. The acetylated proteins were detected by autoradiography after exposure at -80 ℃ for five days. Equal loading of the proteins was confirmed by Coomassie blue staining. MBP-AtJAZ6 was used as an acetylation substrate of HopZ1a.

The result was repeated three times with consistent results.

Z1a (32-369)	+		+		+		+	
Z1a(C216A) (32-369)		+		+		+		+
MBP-AtJAZ6							+	+
GmZINP2	+	+						
GmZINP2(1-144)			+	+				
GmZINP2(145-333)					+	+		



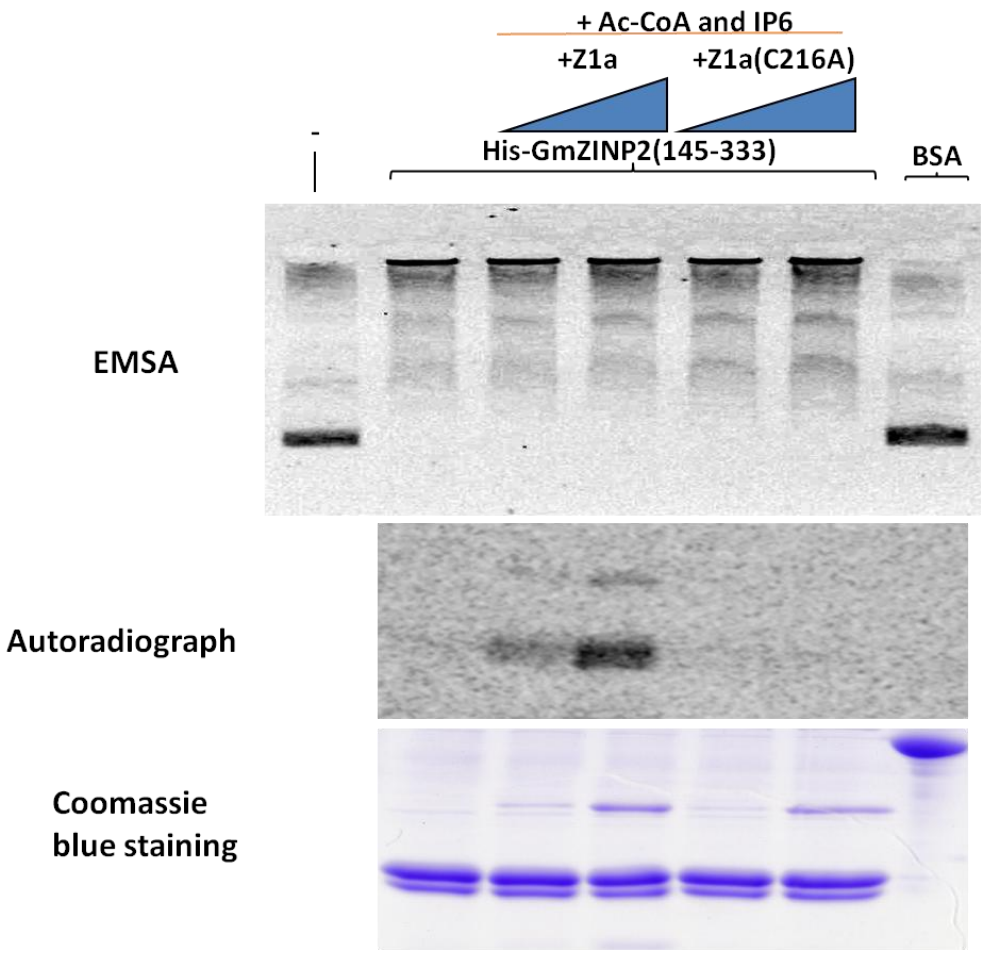
The DNA binding activity of GmZINP2 is not affected by HopZ1a-mediated acetylation

Since HopZ1a acetylated the C-terminus of GmZINP2, which is required for DNA binding activity, I examined whether HopZ1a-mediated acetylation affects the DNA binding activity of GmZINP2. Purified GmZINP2(145-333) was acetylated *in vitro* by HopZ1a before being co-incubated with Litmus 28i for the EMSA assay. HopZ1a(C216A) was used as a negative control. I showed that HopZ1a-mediated acetylation on GmZINP2(145-333) did not affect its DNA binding activity on Litmus 28i (Figure 2.9). In order to confirm that the formation of large nucleoprotein complex is specific, equal molar ratio of BSA protein was added and no DNA binding activity could be observed.

Figure 2.9. Acetylation of GmZINP2 by HopZ1a does not affect its DNA binding activity.

0.1 ρmol Litmus 28i plasmid was incubated with GmZINP(145-333) at molar ratios of GmZINZP2/DNA of 1200 (lane 2-6). GmZINP2(145-333) was acetylated by HopZ1a using C12 acetyl-CoA before incubation when necessary. Nucleoprotein complexes were analyzed on 0.6% agarose gels and visualized by ethidium bromide staining. Same molar ratio of BSA/DNA was included to confirm the specificity of this interaction (lane 7). Acetylation of GmZINP2(145-333) was confirmed by autoradiography and C14 acetyl-CoA was used instead. Equal loading of proteins was confirmed by Coomassie blue staining.

The experiment was repeated twice with consistent results.


+ Ac-CoA and IP6
+Z1a
+Z1a(C216A)
-
His-GmZINP2(145-333)
BSA
EMSA
Autoradiograph
Coomassie
blue staining

HopZ1a affects GmZINP2-GmMYB56 interaction

Since HopZ1a did not affect the DNA binding activity of GmZINP2, I tested an alternative hypothesis. As chromatin remodeling protein was often found to function as a complex to regulate gene transcription, my colleague Dr. Huanbin Zhou carried out yeast-two-hybrid assay and identified the interacting partners of GmZINP2 in soybean. One of GmZINP2-interacting proteins is GmMYB56, which was selected for further study as MYB class transcription factor was previously shown to regulate defense (Ambawat et al., 2013).

I first confirmed that the interaction between GmZINP2 and GmMYB56 is specific and no interaction between HopZ1a and GmMYB56 was observed in yeast-two-hybrid assay (Figure 2.10). Next, I showed that GmZINP2(145-333) interacted with GmMYB56 by GST pull down assay (Figure 2.11). Since the DNA binding activity of GmZINP2 was not affected by HopZ1a-mediated acetylation. I investigated whether the interaction between GmZINP2-GmMYB56 was affected by HopZ1a. Purified GST-GmZINP2(144-333) was immobilized on glutathione-agarose beads and used to pull down GmMYB56. Increasing concentration of tag-free HopZ1a or HopZ1a(C216A) were included in the reaction to study the effect of HopZ1a on GmZINP2(144-333) and GmMYB56 interaction *in vitro*. I showed that the presence of HopZ1a increased the interaction between GmZINP2(145-333) and GmMYB56. In the contrary, lowered interaction between GmZINP2(145-333) and GmMYB56 was observed when HopZ1a(C216A) was included (Figure 2.12).

Figure 2.10. GmMYB56 interacts with GmZINP2 but not HopZ1a.

Yeast strain AH109 was transformed with pGBKT7 (BD) carrying GmMYB56 or HopZ1a and pGADT7 (AD) carrying HopZ1a or GmZINP2. Transformants were selected on SD-2 plates, and then individual colonies were examined for growth on the selective SD-4 plates at 30°C for 5 days.

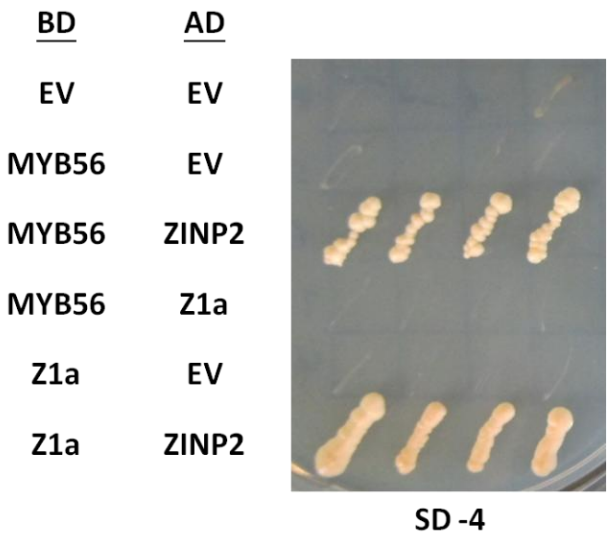


Figure 2.11. GmZINP2(145-333) interacts with GmMYB56.

His tagged GmMYB56, GST and GST-GmZINP2(145-333) proteins were expressed in *E. coli*. Pull down of GmMYB56 by GmZINP2(145-333) was determined by western blotting before (input) and after affinity purification (pull-down) using anti-His antibody (Santa Cruz Biotechnology). The protein abundances of GST and GST-GmZINP2(145-333) on the affinity resins after washes were detected by Coomassie blue staining. The experiment was repeated once with consistent result.

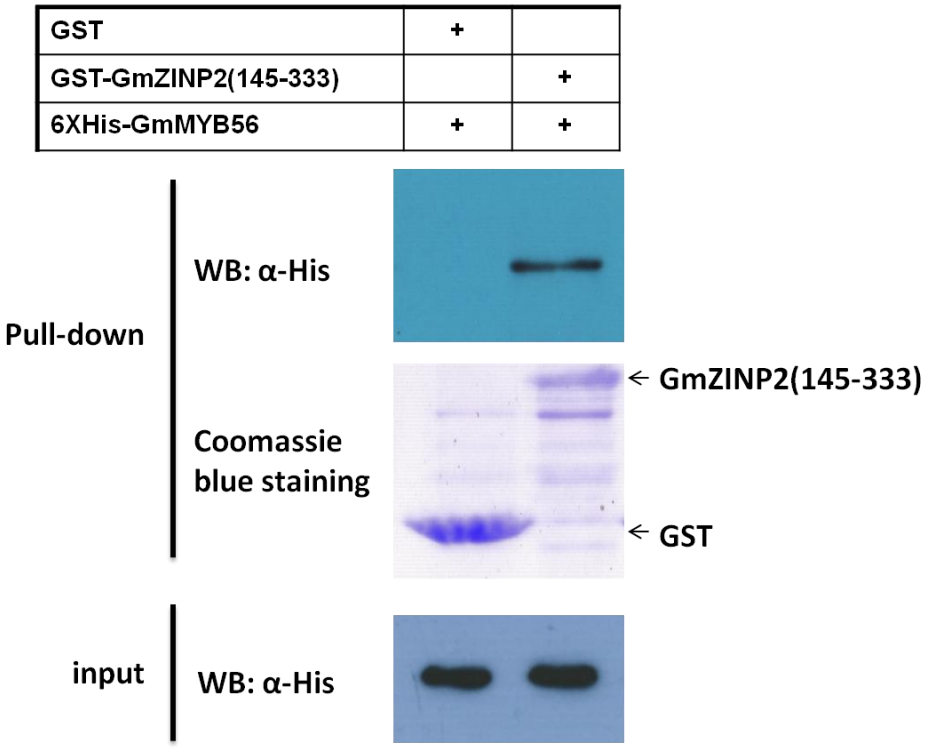
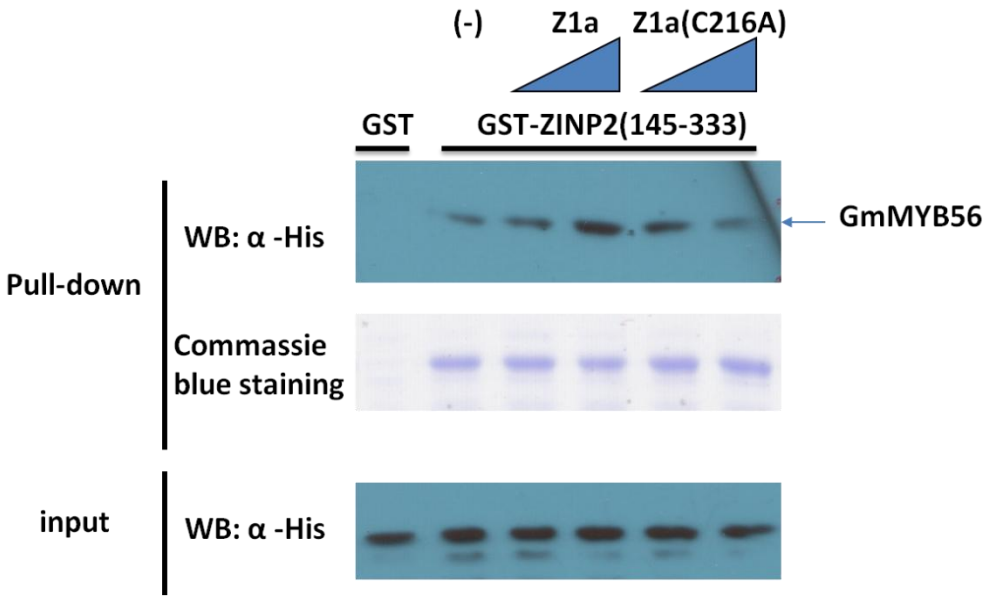


Figure 2.12. HopZ1a affects GmZINP2-GmMYB56 interaction.

His tagged GmMYB56, GST, GST-GmZINP2(145-333), HopZ1a and HopZ1a(C216A) proteins were expressed in *E. coli*. Pull down of GmMYB56 by GmZINP2(145-333) was determined by western blotting before (input) and after affinity purification (pull-down) using anti-His antibody (Santa Cruz Biotechnology). Increasing amount of tag free HopZ1a and HopZ1a(C216A) proteins were added to study the effect of HopZ1a on GmZINP2(145-333)-GmMYB56 interaction. The protein abundances of GST and GST-GmZINP2(145-333) on the affinity resins after washes were detected by Coomassie blue staining.

The experiment was repeated more than three times with consistent results.



The GmZINP2 homolog in Arabidopsis (at4g22360) is not required for defense against *Pto*.

There are three homologs of *GmZINP2* in Arabidopsis and *at4g22360* is the closest homolog of *GmZINP2* (Figure 2.13). In order to study a potential defense function of *at4g22360* in Arabidopsis defense, transgenic plant over-expressing *at4g22360* was generated. I examined the bacterial multiplication of *Pto*DC3000 and the T3SS-defective mutant *hrcC* in wild-type and transgenic Arabidopsis expressing at4g22360. No difference in bacterial multiplication was observed (Figure 2.14).

Figure 2.13. Phylogeny of GmZINP2 homologs in Arabidopsis and soybean.

The phylogenetic tree was constructed using amino acid sequence alignment generated with the ClustalW algorithm using the neighbor-joining method. The evolutionary distance was calculated based on substitution of amino acid per site. At4g22360 is the closest homolog of GmZINP2 and the other two Arabidopsis homologs at3g19080 and at1g49520 form a separate cluster.

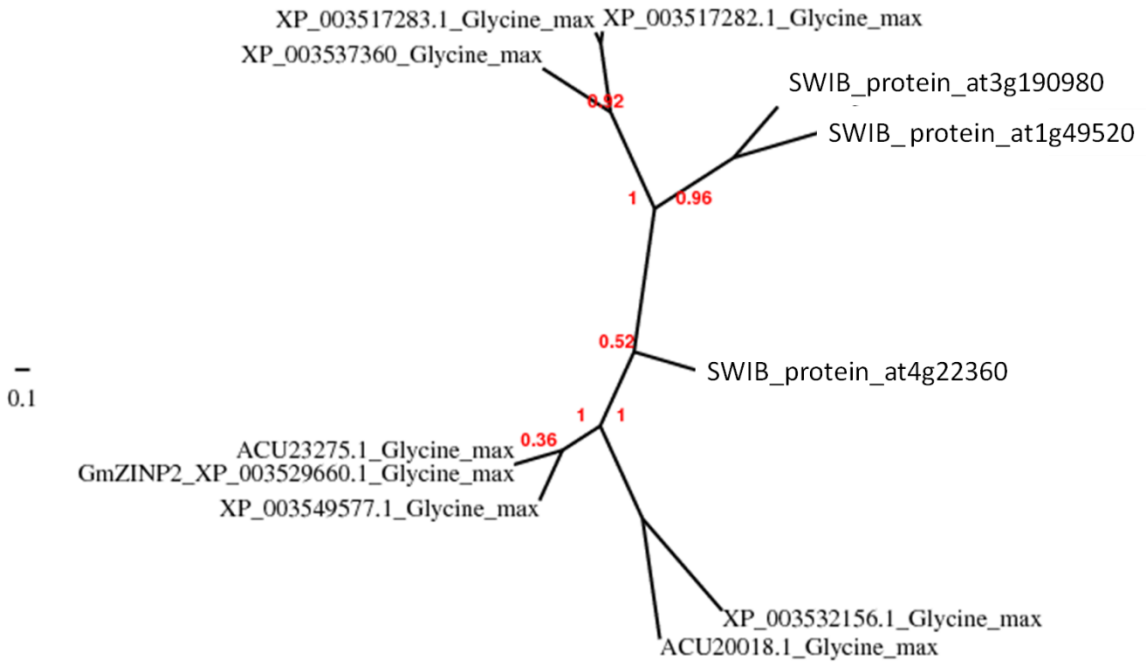
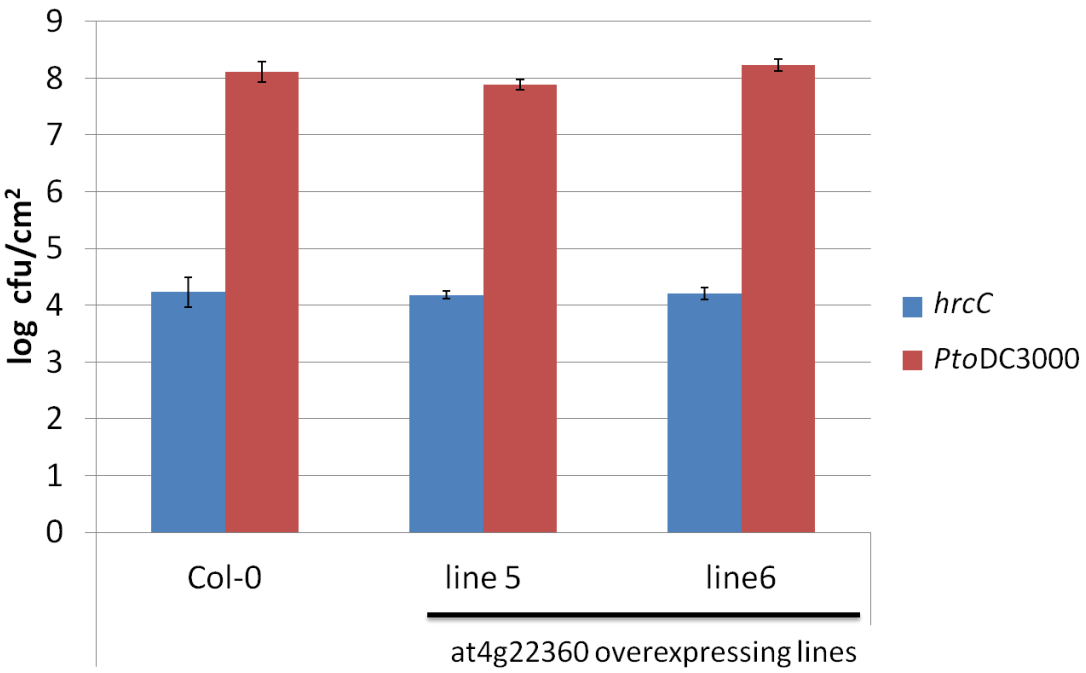


Figure 2.14. A GmZINP2 homolog at4g22360 is not required for Arabidopsis defense against *Pto*DC3000.

Five-week old leaves of wild-type Arabidopsis or *at4g22360* over-expressing plants were infiltrated with *Pto*DC3000 or the T3SS-defective mutant *hrcC* at OD_{600} = 0.0001 ($1x10^5$ cfu/mL) and 0.005 ($5x10^6$ cfu/mL) respectively. Bacterial populations were determined at 3 days post inoculation (dpi). Average colony forming units per square centimeter (cfu/cm^2) and standard deviations (as error bars) are presented.

The experiment was repeated twice with similar results.



DISCUSSION

Chromatin-associated proteins are involved in various processes through the regulation of chromatin structure and function. Even though these proteins have been shown to play a role in plant innate immunity, the underlying mechanisms remain largely unknown. In addition, none of chromatin remodeling proteins has been shown to be a target of T3SE; therefore, the discovery of HopZ1a-GmZINP2 interaction in soybean is novel. Interestingly, GmZINP2 is a modular protein with an N-terminal DEK-C domain and a C-terminal SWIB domain. In human, DEK has been shown to bind DNA with a preference on supercoiled form of DNA and change DNA topology (Waldmann et al., 2003; Kappes et al., 2004b; Bohm et al., 2005). The DEK3 protein in plants regulates salt tolerance, possibly through interaction with H3 and H4 and modulation of nucleosome occupancy at target loci (Waidmann et al., 2014b). Surprisingly, even though DNA binding activity could be observed from GmZINP2, the DEK-C containing truncate i.e. GmZINP2(1-144) barely showed any DNA binding in my EMSA assay. Indeed, the use of Litmus 28i in the EMSA assay only showed that GmZINP2 has a general DNA binding activity, whether GmZINP2(1-144) can bind DNA in a sequence specific manner is waiting to be discovered. Alternatively, the DNA binding activity of DEK-C can be regulated by polymerization and post-translational modifications including phosphorylation, acetylation and ribosylation (Kappes et al., 2004c; Kappes et al., 2004a; Soares et al., 2006; Kappes et al., 2008). However a potential HopZ1a-mediated acetylation of DEK-C domain in GmZINP2 is unlikely based on the GST pull down and

in vitro acetylation data. Further experiments are needed to confirm the function of DEK-C domain in GmZINP2.

The SWIB domain is found in BAF60b of SWI/SNF complex in human (Nomoto et al., 1997). Homology remodeling between SWIB and MDM2 domain suggests that these two domains may adopt a similar fold and function as a protein-protein interaction platform (Bennett-Lovsey et al., 2002). Information on the SWIB domain containing proteins in plants is rather limited. A group of low molecular weight SWIB domain-containing proteins are localized in the nucleoid of organelles and function to remodel nucleoid architecture (Melonek et al., 2012). Even though SWIB domain proteins are generally present exclusively in eukaryotes, it is also produced by the obligate bacteria pathogen *Chlamydia* as a fusion protein to the topoisomerase. This fusion protein is likely acquired from the eukaryotic host to function during cell cycle progression (Stephens et al., 1998). Consistent with a role of the SWIB domain in mediating protein-protein interaction, I showed that the C-terminus of GmZINP2 interacts with HopZ1a and GmMYB56. Intriguingly, HopZ1a enhances the interaction between GmZINP2 and GmMYB56. As MYB transcription factors have been implicated in defense (Ambawat et al., 2013), it is tempting to speculate that GmMYB56 regulates the expression of defense-associated genes, and this activity is modulated by GmZINP2. The demonstration of DNA binding by GmZINP2(145-333) is also interesting. However, whether the SWIB domain is responsible for DNA binding still requires further validation. In particular, GmZINP2(145-333) purified from *E. coli* always migrates as two bands. It will be

interesting to see whether these two bands correspond to post-translational modification(s) on GmZINP2.

GmZINP2 is a negative regulator of defense involved in both PTI and ETI, at least HopZ1a-specific ETI response. Assuming that GmZINP2, and possibly GmMYB56, regulate the expression of defense-related genes; the next logical question is which genes are under the regulation of GmZINP2. Examples from chromatin remodeling proteins including BRM and SWI3C suggest that phytohormone signaling is a potential target in mediating global transcriptomic changes (Walley et al., 2008; Sarnowska et al., 2013). Here I showed that the SA-defense marker gene *GmPR-1* was down-regulated by over-expression of *GmZINP2* upon bacterial infection. However whether such a repression is directly related to the SA pathway or indirectly from crosstalk among other phytohormone signaling is waiting to be discovered. There are three homologs of *GmZINP2* in Arabidopsis. To my surprise, no apparent defense function was observed in the transgenic plant over-expressing the closest homolog *at4g22360*. It is possible that the *GmZINP2* homologs in Arabidopsis may have diverged for a completely different function. It is also possible that the other two *GmZINP2*-like genes regulate defense response in Arabidopsis.

In summary, I reported a novel negative regulator of defense against *Pgy* in soybean. GmZINP2 contains two domains that are associated with chromatin remodeling function. My experiments confirmed that it has DNA binding activity. HopZ1a acetylates GmZINP2 and affects the interaction between GmZINP2 and a MYB class transcription factor GmMYB56. This finding provides mechanistic insight into how chromatin

remodeling proteins may regulate plant innate immunity and how bacterial pathogens use T3SEs to manipulate these regulatory proteins.

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