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Understanding Effector Secretion and Function of two Proteobacteria: *Pseudomonas syringae* and *Candidatus* Liberibacter asiaticus

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

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by

Eva Hawara

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Dissertation Committee:
Dr. Wenbo Ma, Chairperson
Dr. Georgios Vidalakis
Dr. Xuemei Chen

The Dissertation of Eva Hawara is approved:

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Committee Chairperson

University of California, Riverside

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DEDICATION

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ABSTRACT OF THE DISSERTATION

Understanding Effector Secretion and Function of two Proteobacteria: *Pseudomonas syringae* and *Candidatus* Liberibacter asiaticus

by

Eva Hawara

Doctor of Philosophy, Graduate Program in Microbiology
University of California, Riverside, March 2020
Dr. Wenbo Ma, Chairperson

Gram-negative bacteria employ secretion systems to deliver virulence factors, such as effectors, to target plant immunity in hosts. My research focuses on secreted effectors from plant pathogenic bacteria *Pseudomonas syringae* and *Candidatus* Liberibacter asiaticus.

In chapter one, I focused on *P. syringae* type III secreted effector HopZ1a. I confirmed the relationship between HopZ1a-mediated acetylation and subsequent degradation of its target JAZs (JAZ10) in *Arabidopsis thaliana*. Next, I determined decreased susceptibility in JAZ10 plants mutated in acetylation sites during infection, indicating HopZ1a-mediated acetylation and subsequent degradation of JAZ10 affects bacterial growth. My findings in this chapter highlight the significance of HopZ1a's acetylation modification of JAZs.

In chapter two, I optimized the use of *C*Las Sec-delivered effector 1 (SDE1) (CLIBASLIA_05315) for direct tissue blot immunoassay (DTBIA). I also

generated *Liberibacter crescens* (*L. crescens)* cell and lipopolysaccharide (LPS) specific antibodies to serve as a cocktail primary antibody for enzyme-linked immunosorbent assay (ELISA). Furthermore, *L. crescens* LPS structural analysis by the Complex Carbohydrate Research Center (CCRC) revealed the presence of very long chain fatty acid (VLCFA 27OHC28:0). It is possible this VLCFA is required for culturing of Liberibacters.

In chapter three, I generated a functional model system to confirm *C*Las predicted secreted effectors using *L. crescens*. Our lab generated a list of predicted secreted effectors of *C*Las and we focused on some for detection biomarkers, and functional work was performed on one effector SDE1 (CLIBASIA_05315). Using this foundation of knowledge, I used SDE1 as my test subject for secretion in *L. crescens*. My research shows that *L. crescens* can serve as a tool to confirm and possibly study *C*Las secreted effectors.

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GENERAL INTRODUCTION

Plant immunity

Plants, unlike mammals, lack mobile defender cells and a somatic adaptive immune system; therefore, they rely on the innate immunity of each cell and on systemic signals emanating from infection sites (Jones and Dangl, 2006). Once a pathogen has bypassed the plants multiple layers of protection and reached the host plasma membrane, the first branch of the plant innate immune system will be activated due to the recognition of microbial molecules, referred to as pathogen/microbe associated molecular pattern (PAMPs/MAMPs), via pattern recognition receptors (PRRs) which leads to a signal transduction cascade, and eventually pattern triggered immunity (PTI) (Chisholm et al., 2006; Zipfel, 2009). PTI involves the induction of MAPK signaling cascades, calcium flux, production of nitric oxide and reactive oxygen species, and the activation of stress-specific WRKY transcription factors (Asai et al., 2002; Nurnberger et al., 2004; He et al., 2006). PTI-mediated basal immunity effectively restricts the growth of the vast majority of potential pathogens, and is largely responsible for the general health of most plants (Ma and Guttman, 2008).

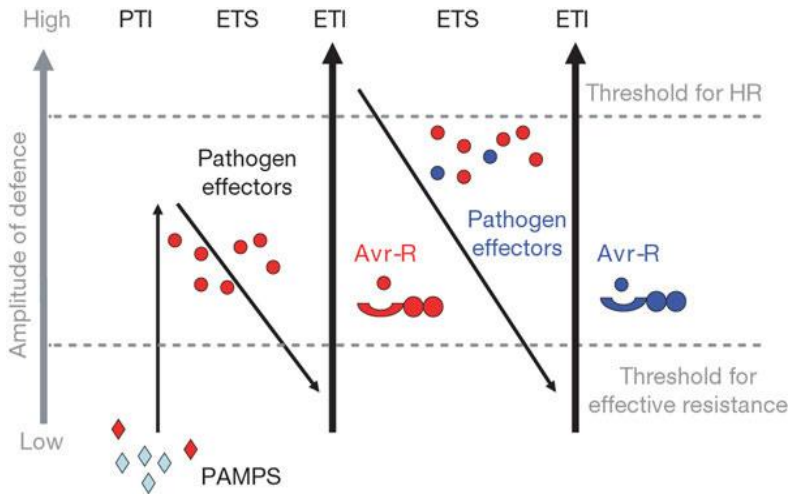
Successful pathogens deploy effectors that contribute to pathogen virulence by interfering with PTI resulting in effector-triggered susceptibility (ETS) (Jones and Dangl, 2006). Plants perceive such effectors through additional receptors, typically nucleotide-binding leucine-rich repeat (NB-LRR) proteins, to mount a second layer of defense called effector-triggered immunity (ETI) frequently

associated with the development of a localized programmed cell death called hypersensitive response (HR) (Boller and He, 2009) **(Figure G1a)**. Pathogens may then respond by modifying or even losing the T3SE so that they are no longer detected (Jones and Dangl, 2006). In this manner, bacterial T3SEs and plant resistance (R) proteins are engaged in a classic co-evolutionary arms race (Ma and Guttman, 2008). NB-LRR proteins can recognize pathogen effectors either directly by physical association or indirectly where an effector induces a change in an accessory protein enables the accessory to be recognized by the NB-LRR protein (van der Hoorn and Kamoun, 2008). The 'guard' model postulates that NB-LRR proteins guard an accessory protein (or guarded/decoy) that is targeted and modified by pathogen effectors (Dangl and Jones, 2001; Dodds and Rathjen, 2010).

Recent research showing PTI and ETI share largely overlapping signaling networks and downstream responses led to a new layered paradigm of plant immunity. The layered immunity system consists of a recognition layer, a signal-integration layer, and a defense-action layer (Wang et al., 2019) **(Figure G1b)**. The recognition layer includes cell surface pattern recognition receptors (PRRs) that can recognize apoplastic effectors, microbe-associated molecular patterns (MAMPs; e.g., flagellin or pathogen cell wall fragments), or damage-associated molecular patterns (DAMPs; e.g., plant cell wall fragments or ATP). The recognition layer also includes intracellular receptors that can recognize intracellular effectors by direct binding, recognize complexes of host proteins with

effectors, or recognize host proteins that have been modified by effectors. The signal-integration layer accepts signals from the recognition layer creating a complex network including phosphorylation, stabilization, degradation and signaling by plant hormones. The signal-integration layer accepts incoming signals from neighboring cells and distant tissues and outputs a tuned set of signals to the defense action layer. defense-action layer consists of diverse actions (such as programmed cell death production and secretion of antimicrobial proteins) that can be tuned to provide protection against one or more specific pathogens, as well as modulate interactions with the ambient microbiome.

a


High
PTI
ETS
ETI
ETS
ETI
Threshold for HR
Amplitude of defence
Pathogen effectors
Avr-R
Pathogen effectors
Avr-R
Threshold for effective resistance
Low
PAMPS

b

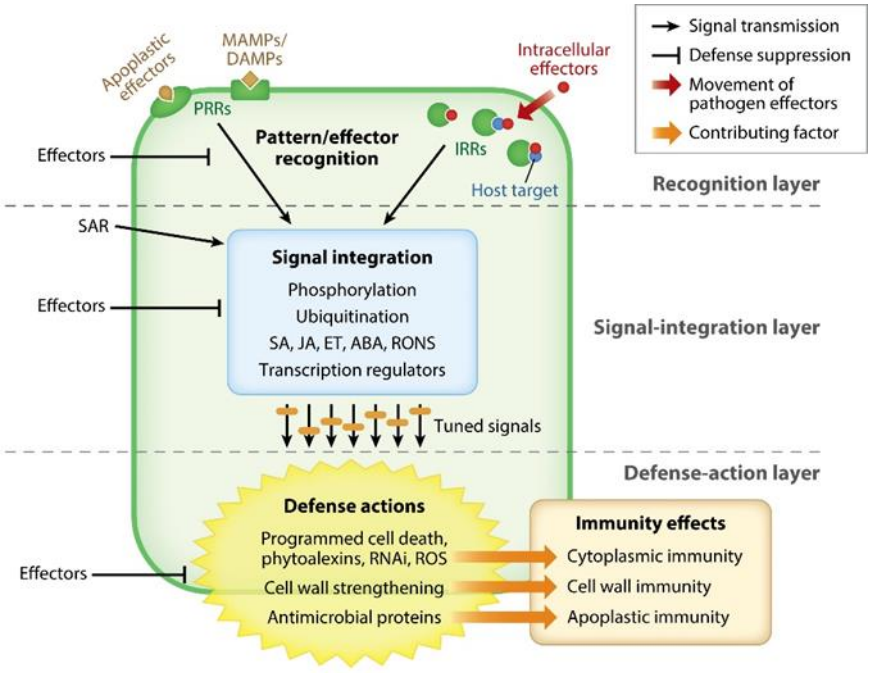

Signal transmission
Defense suppression
Movement of pathogen effectors
Contributing factor
Apoplastic effectors
MAMPs/ DAMPs
Intracellular effectors
PRRs
Pattern/effector recognition
IRRs
Host target
Effectors
Recognition layer
SAR
Signal integration
Phosphorylation
Ubiquitination
SA, JA, ET, ABA, RONS
Transcription regulators
Effectors
Signal-integration layer
Tuned signals
Defense-action layer
Defense actions
Programmed cell death, phytoalexins, RNAi, ROS
Cell wall strengthening
Antimicrobial proteins
Immunity effects
Cytoplasmic immunity
Cell wall immunity
Apoplastic immunity
Effectors

Figure G1. **Evolving models of plant immunity a)** Zigzag model illustrates the quantitative output of the plant immune system. Plants detect (MAMPs/PAMPs, red diamonds) via PRRs to trigger PAMP-triggered immunity (PTI). Pathogens deliver effectors that interfere with PTI, resulting in effector-triggered susceptibility (ETS). One effector (indicated in red) is recognized by an NB-LRR protein, activating effector-triggered immunity (ETI) often inducting of hypersensitive cell death (HR)(Jones and Dangl, 2006) **b)** Layered immunity consists of three layers, a recognition layer, a signal-integration layer, and a defense-action layer (Wang et al., 2019).

Bacterial Secretion systems

Microbes utilize their secretory mechanisms to facilitate host-microbe crosstalk via highly specialized secretory proteins called effectors. In cells from all three domains of life, more than one-third of the proteome is secreted across or inserted into biological membranes (Papanikou et al., 2007). Many bacterial associations and functions rely upon protein secretion, whether the interactions are mutualistic or pathogenic; therefore, bacteria have evolved specialized secretory machinery to fulfil this requirement (Tseng et al., 2009) **(Figure G2)**. The general secretory (Sec) pathway and two-arginine translocation (Tat) pathway, are universal across the tree of life and serve to translocate proteins from the cytoplasm to the periplasm in gram-negative bacteria and the extracellular space in gram-positive bacteria (Cao and Saier, 2003; Müller, 2005; Tripathy and Bhowmick, 2014) . In order to overcome the hurdle of their bilayer membrane, gram-negative bacteria have evolved secretory systems that can be divided into two categories: Sec/Tat-dependent and Sec/Tat-independent. In Sec-dependent systems, once the proteins are delivered to the periplasm, they depend upon the Type II, Type V, Type IX or less commonly the Type IV or Type I systems to be ejected to the extracellular milieu (Saier, 2006; Tseng et al., 2009; McBride and Zhu, 2013; Sato et al., 2013). In the Sec-independent systems, proteins are delivered from the cytoplasm to the extracellular space in one step via Type I, Type III, Type IV, Type VI and Type VII (utilized by mycobacteria) (Abdallah et al., 2007; Tseng et al., 2009).

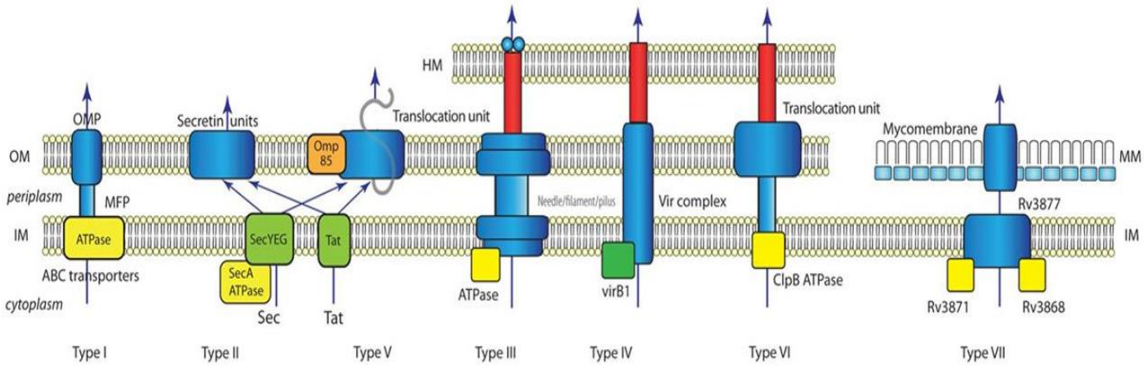


Figure G2. Summary of known bacterial secretion systems. In this simplified view only the basics of each secretion system are sketched. HM: Host membrane; OM: outer membrane; IM: inner membrane; MM: mycomembrane; OMP: outer membrane protein; MFP: membrane fusion protein. ATPases and chaperones are shown in yellow (Tseng et al., 2009).

Pathogen effectors

Once translocated into the plant cytoplasm, effectors can traffic to different subcellular compartments, including organelles and various membrane compartments (Win et al., 2012). Essentially, any effector activity that would increase the fitness of the microbe, its ability to colonize the host plant, and/or spread to other hosts could potentially evolve (Win et al., 2012). Some effectors, particularly bacterial T3SS effectors, can function as enzymes that biochemically modify host molecules, typically impeding their function or eliminating them (Cunnac et al., 2009; Deslandes and Rivas, 2012). The enzymatic activities of effectors are diverse and include protease, hydrolase, phosphatase, kinase, transferase, and ubiquitin ligase activities (Shao, 2003; Abramovitch et al., 2006; Janjusevic, 2006; Fu et al., 2007; Lee et al., 2012; Rodríguez-Herva et al., 2012; van Damme et al., 2012). Other effectors act by binding host proteins to modulate their functions. Many such effectors inhibit plant enzymes such as kinases, proteases, glucanases, and peroxidases (Tian et al., 2004, 2007; Rooney, 2005; Damasceno et al., 2008; Xiang et al., 2008; Song et al., 2009; Clark et al., 2018). Another group of effectors (*Xanthomonas* TAL effectors) have evolved to bind nucleic acids acting as modulators of gene expression (Duan et al., 1999; Boch et al., 2009; Domingues et al., 2010; de Souza et al., 2012). In addition to actively inhibiting PRR-mediated responses, some pathogens have evolved mechanisms to circumvent immune detection by sheltering or masking PAMPs perceived by PRRs (Toruño et al., 2016). Some plant proteins

biochemically modify the effectors, contribute to effector maturation inside the plant cytoplasm, or serve as cofactors that form biochemically active complexes with the effector (Win et al., 2012).

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Chapter 1

Understanding the relationship between acetylation and degradation of *Arabidopsis* JAZs during *Pseudomonas syringae* infection

ABSTRACT

Pseudomonas syringae type III secreted effector HopZ1a targets and acetylates multiple *Arabidopsis* Jasmonate ZIM-domain (JAZ) proteins, leading to their degradation. HopZ1a can also partially rescue the virulence defect of a *Pseudomonas syringae* mutant that lacks the production of coronatine (COR), a jasominc acid (JA)-mimicking phytotoxin produced by a few *P. syringae* strains. JAZs are key negative regulators in jasmonate signaling; by degrading JAZs HopZ1a activates JA signaling and promotes bacterial multiplication. Preliminary (unpublished) data from Dr. Shushu Jiang, in the Wenbo Ma laboratory, identified potential acetylation sites in HopZ1a's target JAZ10 (AT5G13220 from *Arabidopsis thaliana* eco. Col-0). Using *Nicotiana benthamiana* co-expression of HopZ1a with JAZ10, and acetylation site mutants JAZ10$^{(S62A, S64A, T74A)}$ and JAZ10$^{\Delta(N61\text{-}P75)}$, she proceeded to confirm reduction in degradation of the acetylation mutants. In this chapter, I investigated the relationship between acetylation and degradation by infecting transgenic *Arabidopsis* lines over-expressing JAZ10, JAZ10$^{(S62A, S64A, T74A)}$ and JAZ10$^{\Delta(N61\text{-}P75)}$ with *P. syringae* expressing HopZ1a. I used *P. syringae* infection to determine susceptibility in

jaz10/*zar1-1* expressing JAZ10$^{(S62A, S64A, T74A)}$ or JAZ10$^{\Delta(N61\text{-}P75)}$. My findings from this research highlight the significance of HopZ1a's acetylation of JAZs.

INTRODUCTION

Pseuodomonas syringae is a proteobacteria in the same subgroup as a number of important pathogens, including animal pathogens: *Escherichia*, *Salmonella*, *Shigella*, and *Yersinia spp*. and plant pathogenic *Erwinia*, *Pantoea*, *Xanthomonas*, and *Xylella* (Gupta, 2000). The genus *Pseudomonas* is notable because it contains the clinically important human pathogen *Pseudomonas aeruginosa*, as well as the agriculturally important plant pathogen *Pseudomonas syringae* (Hirano and Upper, 2000). *P. syringae* was isolated in 1902 from an infected lilac by M. W. Beijerinck (Young, 1991). Today, we understand *P. syringae* to be a collection of biochemically related strains, that can have distinct plant host ranges (Bretz and Hutcheson, 2004). *P. syringae* consists of over 50 pathovars infecting a wide array of plants including, but not limited to fruits, vegetable and ornamentals (Gardan et al., 1999; Xin and He, 2013). A typical symptom of *P. syringae* infection is an initial "water soaking" at the site of infection (indicative of altered membrane physiology) followed by slowly developing cell death (PCD), and in the case of exotoxin producers, a spreading chlorosis (yellowing of the tissue due to chlorophyll breakdown) (Bender et al., 1999; Lindow and Brandl, 2003; Bretz and Hutcheson, 2004). The diseases that *P. syringae* strains cause range from foliar spot diseases to blights, stripes,

and cankers (Agrios, G.N., 2005). The disease usually does not kill the plant but diminishes the yield and marketability of the product. *P. syringae* is transmitted mainly by rain and wind. The bacteria can survive on leaf surfaces as epiphytes without causing disease, and then enter leaves either through natural openings, like stomata, or through wounds proliferating in the intercellular spaces (the apoplast) (Jones and Dangl, 2006; Mohr et al., 2008). When bacterial population reaches high densities then it causes visible disease symptoms (Hirano and Upper, 2000).

Once the plant interior has been breached, microbes are faced with another obstacle: the plant cell wall, a rigid, cellulose-based support surrounding every cell; thus, penetration of the cell wall exposes the host plasma membrane to the microbe (Chisholm et al., 2006). Many gram-negative pathogenic bacteria employ a type III secretion system (T3SS) that provides them with the unique mechanism to bypass the extracellular milieu, and inject bacterial effector proteins directly into the host cell cytoplasm (Coburn et al., 2007). For an operating T3SS, a complex of proteins at the tip of this “needle structure” contribute to create a pore into the target host cell membrane and form a channel connecting the bacteria with the host cell (Mueller et al., 2008). T3SS pathway is encoded by *hrp* (HR and pathogenicity) and *hrc* (HR and conserved) genes (Bogdanove et al., 1996). The Hrc proteins direct secretion of T3SS substrates across the bacterial envelope, whereas a subset of the Hrp proteins are themselves secreted by the T3SS and direct the translocation of effectors

through host cell barriers (Alfano and Collmer, 2004). *P. syringae* has also been widely used as a model system for understanding plant-bacterial interactions. *P. syringae* pv. *tomato* strain DC3000 (*Pto*DC3000) causes bacterial speck of tomato, a worldwide, economically significant disease that is representative of numerous bacterial plant diseases (Wilson et al., 2002; Buell et al., 2003). *Pto*DC3000 is also a pathogen of the model plant *Arabidopsis thaliana* (Whalen et al., 1991). Importantly, the pathogenicity of *Pto*DC3000 resembles that of most animal and plant pathogens in the gammaproteobacterial class, which rely on the T3SS to inject virulence effector proteins into host cells (Cornelis and Van Gijsegem, 2000). Whether we are studying the virulence of *P. syringae* or using it as a model to study other pathogens/effectors, understanding these bacteria will benefit science in a multitude of avenues.

Plants are continuously exposed to microbes, and their sessile nature adds another level of difficulty as they must continuously integrate both biotic and abiotic signals from the environment (Jones and Dangl, 2006). Furthermore, plants often deal with simultaneous or subsequent invasion by multiple aggressors, which can influence the primary induced defense response of the host plant (Van der Putten et al., 2001; Bezemer and Vandam, 2005; Stout et al., 2006). Hence, plants need regulatory mechanisms to effectively adapt to changes in their hostile environment. Cross talk between induced defense-signaling pathways is thought to provide the plant with such a powerful regulatory potential (Pieterse et al., 2012). Cross talk helps the plant to minimize energy

costs and create a flexible signaling network that finely tunes defense response depending on the invaders (Reymond and Farmer, 1998; Bostock, 2005). Jasmonic acid (JA) and salicylic acid (SA) are major defense-related phytohormones produced within plants that govern diverse physiological processes (Berens et al., 2017). Other phytohormones, such as ethylene (ET), abscisic acid (ABA), auxin, gibberellins (GAs), cytokinins (CKs), and brassinosteroids (BRs), are also involved in defense responses (Shigenaga and Argueso, 2016; Berens et al., 2017). In *A. thaliana*, although there are exceptions, JA is a positive regulator of immunity against necrotrophic pathogens that actively kill hosts to acquire nutrients and herbivore defense, whereas SA is a positive regulator of immunity against biotrophic pathogens that feed on living hosts as well as against hemibiotrophs that show a biotrophic phase in the early stage of infection (Thomma et al., 1998; Wildermuth et al., 2001; van Wees et al., 2003; Glazebrook, 2005; Liu et al., 2016; Berens et al., 2017). Previously an antagonistic relationship between SA and JA/ET pathways in response to pathogens was accepted (Spoel et al., 2009; Van der Does et al., 2013). Further analysis using a mutant that is defective in SA, JA and ET pathways supported a more synergistic view in that all three hormones contribute positively to defense against various pathogens with one hormone sector makes larger contributions than others in response to a specific infection style (Tsuda et al., 2009; Ma and Ma, 2016a). Despite this wholistic view of plant phytohormones, plethora of evidence shows *P. syringae* strategizes to manipulate JA production. Type III

secreted effector of *P. syringae*, HopX1, activates JA signaling and acts as a cysteine protease to directly hydrolyze JAZs in *Arabidopsis* (Gimenez-Ibanez et al., 2014). In 2015, another type III secreted effector, AvrB, was also shown to induce JAZs degradation (Zhou et al., 2015). Besides pathogens, the symbiotic ectomycorrhizal fungus *Laccaria bicolor* also produces an effector (MiSSP7), which is necessary for the establishment of symbiosis and acts by binding to the PtJAZ6 protein in its host poplar (Plett et al., 2014). Binding of MiSSP7 to PtJAZ6 stabilizes the JAZ protein to suppress JA-dependent defenses that would otherwise attenuate the symbiosis (Pieterse et al., 2014; Plett et al., 2014). The findings that multiple virulence factors, including both toxins and effectors, manipulate the same host targets highlight the importance of JA pathway as a virulence target (Ma and Ma, 2016a). The toxin coronatine produced by the bacterial pathogen *P. syringae* pv. *tomato* strain DC3000 (*Pto*DC3000) is by far the best studied example of a virulence factor that can manipulate the JA pathway (Ma and Ma, 2016a). Structurally mimicking JA-Ile (JA-isoleucine), COR is approximately 1000-fold more effective in inducing the degradation of JASMONATE ZIM-DOMAIN (JAZ) proteins and acts as a robust inducer of JA signaling (Katsir et al., 2008b; Ma and Ma, 2016). In nature, the activation of JA pathway occurs upon a rapid increase in endogenous JA levels, resulting from environmental stimuli or developmental programs, leading to a concomitant increase in JA-Ile via Jasmonic acid-amido synthetase (JAR1) activity (Staswick, 2008; Koo et al., 2009). In the presence of a high JA-Ile level, the F-box protein

CORONATINE-INSENSITIVE 1 (COI1) recruits its targets, JAZ proteins, from their initial binding site, e.g., MYC2, by physical interaction (Chini et al., 2007; Thines et al., 2007; Katsir et al., 2008a, 2008b). JAZ proteins inhibit transcription factors that regulate early JA-responsive genes; moreover, the functional output of increase in JA-Ile is the degradation of the JAZ repressor(s) via the 26S proteasome, thereby allowing transcription factors such as MYC2 to activate expression of JA-responsive genes (Pauwels and Goossens, 2011).

There are 12 proteins of the *Arabidopsis* jasmonate ZIM-domain (JAZ) family that share three conserved sequence regions: the ZIM domain, Jas motif and weakly conserved N-terminal region; however, atypical JAZ13 has divergent domains (Chini et al., 2007, 2016; Thines et al., 2007; Vanholme et al., 2007; Yan et al., 2007; Thireault et al., 2015). The ZIM domain is within the central portion of the proteins and is required for repressing JA signaling and mediating the homo- and hetero-interactions between different JAZ proteins (Vanholme et al., 2007; Chini et al., 2009; Chung and Howe, 2009; Pauwels and Goossens, 2011). The Jas domain at the C-terminus of the proteins mediates the interaction of JAZ with MYC transcription factors and COI1, thereby plays a critical role in the repression of JA signaling and the stability of JAZ proteins (Chini et al., 2007; Thines et al., 2007; Yan et al., 2007). The COI1-JAZ complex, rather than COI1 alone, was found to function as a high affinity JA receptor (Sheard et al., 2010). NOVEL INTERACTOR OF JAZ (NINJA) was discovered to form protein complexes with JAZs and a region within the ZIM domain is sufficient for JAZ1-

NINJA interaction (Pauwels et al., 2010). NINJA also interacts with the co-repressor TOPLESS (TPL) and its homologs TPL-related proteins (TPRs) in the absence of JA (Pauwels et al., 2010). The interaction between NINJA with TPL suggests a transcription repressor activity of NINJA in JA signaling (Pauwels et al., 2010). The aforementioned work provided a mechanistic explanation of how JAZs function as negative regulators of JA signaling: JAZ proteins recruit co-repressor NINJA and TPL/TPR to specific promoters via the interaction with MYC transcription factors, and inhibit the expression of JA responsive genes (Pauwels et al., 2010; Wager and Browse, 2012).

In 2013, Jiang and colleagues reported that *P. syringae's* type III secreted effector, HopZ1a, directly interacts with JAZ proteins of soybean and *Arabidopsis* inducing the degradation of JAZ1 and promoting JA-responsive gene expression during bacterial infection (Jiang et al., 2013). Additionally, HopZ1a could functionally complement the growth deficiency of a *Pto*DC3000 mutant that does not produce coronatine, uncovering another *P. syringae* tactic (Jiang et al., 2013). HopZ1a is from *P. syringae* pv. *syringae* strain A2 and it belongs to the *Yersinia* outer-protein J (YopJ) family of effector proteins, which are found in both animal- and plant-pathogenic bacteria (Zhou et al., 2009). YopJ-like type III secreted effectors share a conserved catalytic core, consisting mostly of three key amino acid residues (histidine, glutamic acid, and cysteine), which is identical to that of clan-CE (C55-family) cysteine proteases (Orth et al., 2000). However, instead of protease activity, YopJ effectors possess acetyltransferase activity and

modify their target protein through acetylation. The acetyltransferase activity depends on the catalytic cysteine residue, suggesting that YopJ effectors may adopt a protease-like catalytic core for a different enzymatic reaction (Zhang et al., 2016; Ma and Ma, 2016b). YopJ effectors acetylate specific lysine, serine and threonine residues of several mitogen-activated protein kinases in animals (Mittal et al., 2006; Mukherjee, 2006). Previously, it was shown that HopZ1a possesses weak cysteine protease activity using a generic substrate *in vitro* (Ma et al., 2006). Interestingly, it also acts as an acetyltransferase and modifies several plant substrates including tubulin (Lee et al., 2012), and pseudokinase HopZ-ETI-deficient 1 (ZED1) (Lewis et al., 2013). A reverse genetic screen revealed that the *Arabidopsis* R protein HOPZ-ACTIVATED RESISTANCE 1 (ZAR1) is required for recognition of HopZ1a in *Arabidopsis* (Lewis et al., 2010). The activation of HopZ1a-triggered immunity of ZAR1 requires ZED1; however, ZED1 has mutations in the aspartate residue of its catalytic loop; therefore, it appears that ZED1 is the decoy in the “guard and decoy” model (van der Hoorn and Kamoun, 2008; Lewis et al., 2013; Ma and Ma, 2016b). HopZ1a with the catalytic cysteine (C216) residue substituted with alanine (HopZ1aC/A) loses the virulence function or HR-triggering activity (Ma et al., 2006; Lewis et al., 2008; Zhou et al., 2009). In 2016, Zhang and colleagues reported that the eukaryotic-specific inositol hexakisphosphate (IP6) serves as a co-factor of HopZ1a and an allosteric switch that controls the association with the acetyl-group donor AcCoA in HopZ1a (Zhang et al., 2016). Interestingly, IP6 has also been observed in the

RTX toxin of *Vibrio cholerae* and toxin A of *Clostridium difficile* where IP6 binding introduces conformational distortions that facilitate the formation of substrate-binding pockets (Lupardus et al., 2008; Pruitt et al., 2009). These results suggest that YopJ effectors have developed a different regulatory strategy using the same eukaryotic ligand, so that the virulence function is enhanced inside the host cells (Zhang et al., 2016).

Analysis of various *jaz* null mutants suggested functional redundancy between family members (Chini et al., 2007; Thines et al., 2007). However, transgenic plants silenced for the expression of JAZ10 were more sensitive than the wild type to JA (Yan et al., 2007). Due to this reason, our lab decided to pursue the association between HopZ1a and JAZ10 to further understand the relationship between acetylation and degradation. Dr. Shushu Jiang performed the initial experiments for this project shown in figures I-IV. In order to determine if JAZ10 is acetylated by HopZ1a, an *in vitro* acetylation was performed showing HopZ1a strongly acetylates multiple JAZs including JAZ10 (Shushu Jiang, 2013). To identify the sites where JAZ10 is acetylated by HopZ1a, Dr. Shushu Jiang performed an *in vitro* acetylation assay followed by mass spectrometry analysis. Results identified three amino acids of JAZ10 acetylated by HopZ1a but not by the catalytic mutant HopZ1aC/A **(Figure I)**. PCR-based mutagenesis was used to mutate these three amino acids (two serines and one threonine) as putative acetylation sites into alanine, creating the mutant JAZ10$^{(S62A,\ S64A,\ T74A)}$. PCR-based mutagenesis was used to generate the second mutant deleting a 15-

amino acids fragment N61-P75 (including S62, S64, T74) called JAZ10$^{\Delta(N61\text{-}P75)}$. To determine if there was reduction in acetylation of JAZ10$^{(S62A,\ S64A,\ T74A)}$ and JAZ10$^{\Delta(N61\text{-}P75)}$ by HopZ1a, Dr. Shushu Jiang performed an *in vitro* acetylation assay and observed a moderate reduction in acetylation compared to JAZ10 but not abolishment likely due to acetylation sites unrelated to HopZ1a virulence activity that are also present in HopZ1aC/A **(Figure II)**. To confirm the mutations in JAZ10$^{(S62A,\ S64A,\ T74A)}$ and JAZ10$^{\Delta(N61\text{-}P75)}$ didn't hinder interaction with HopZ1a, Dr. Shushu Jiang performed *in vitro* pull-down between HopZ1a and JAZ10, JAZ10$^{(S62A,\ S64A,\ T74A)}$, JAZ10$^{\Delta(N61\text{-}P75)}$, or empty vector and she concluded that interaction was not disrupted **(Figure III)**. Next, Dr. Shushu Jiang used *Nicotiana benthamiana* to transiently co-express JAZ10, JAZ10$^{(S62A,\ S64A,\ T74A)}$, JAZ10$^{\Delta(N61\text{-}P75)}$ and HopZ1a or HopZ1aC/A. She observed a significant reduction of JAZ10 protein level in *N. benthamiana* leaves co-expressing HopZ1a, compared to leaves co-expressing HopZ1aC/A. Moreover, JAZ10$^{(S62A,\ S64A,\ T74A)}$ protein level was reduced in *N. benthamiana* leaves co-expressing HopZ1a, compared to leaves co-expressing HopZ1aC/A or JAZ10. Finally, there was no visible reduction in the protein level of JAZ10$^{\Delta(N61\text{-}P75)}$ when co-expressed with HopZ1a, HopZ1aC/A or empty vector **(Figure IV),** suggesting there is a correlation between increasing the amount of mutated acetylated sites and decrease in degradation of JAZ10.

Figure I. **Mass spectrometry analysis reveals JAZ10 predicted acetylation sites**. *In vitro* acetylation between purified HopZ1a or HopZ1aC/A with JAZ10 was performed and the samples subjected to SDS-PAGE. Bands were cut and sent for mass spectrometry analysis. Yellow highlight indicates peptides identified. Green highlight indicates potential acetylation sites. Black underlines JAZ10 sequence. Three circled amino acids in top analysis indicate the three amino acids mutated to generate JAZ10$^{(S62A, S64A, T74A)}$ and the red rectangle surrounding peptide NSDSSAKSRSVPSTP indicate the 15aa deleted to generate JAZ10$^{\Delta(N61\text{-}P75)}$. (Experiment performed by Dr. Shushu Jiang)

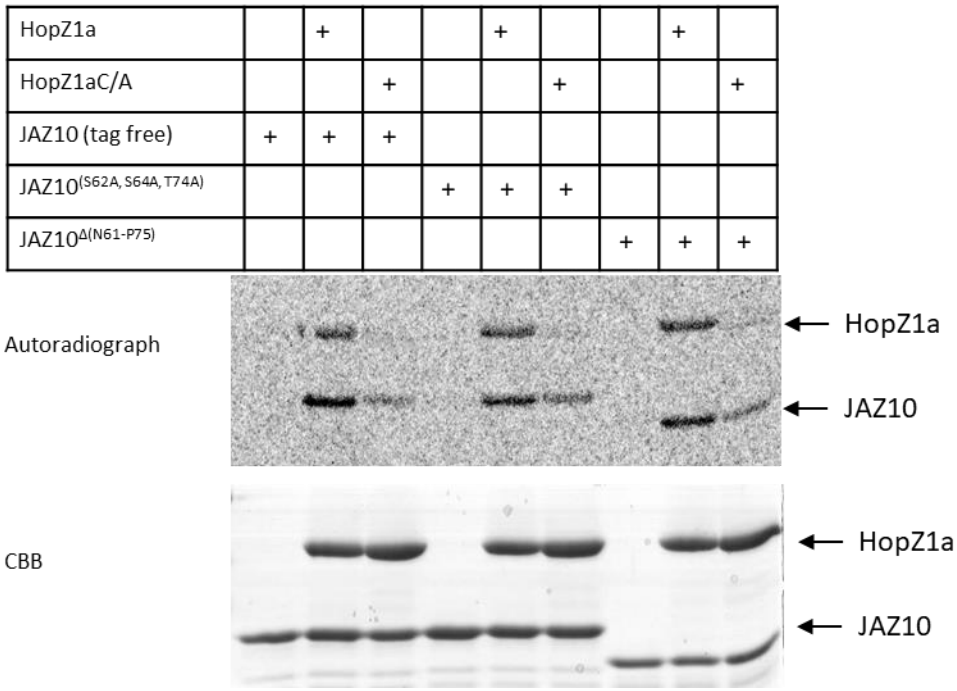


Figure II. Reduction in HopZ1a acetylation of JAZ10$^{(S62A, S64A, T74A)}$ and JAZ10$^{\Delta(N61-P75)}$ *in vitro*. *In vitro* acetylation assay using purified HopZ1a or HopZ1aC/A incubated with purified JAZ10, JAZ10$^{(S62A, S64A, T74A)}$, and JAZ10$^{\Delta(N61-P75)}$. The reactions were then subjected to SDS-PAGE and acetylated proteins were detected by autoradiography. After autoradiography, the protein gels were removed from the filter paper and stained with Coomassie brilliant blue (CBB) as a loading control. (Experiment performed by Dr. Shushu Jiang)

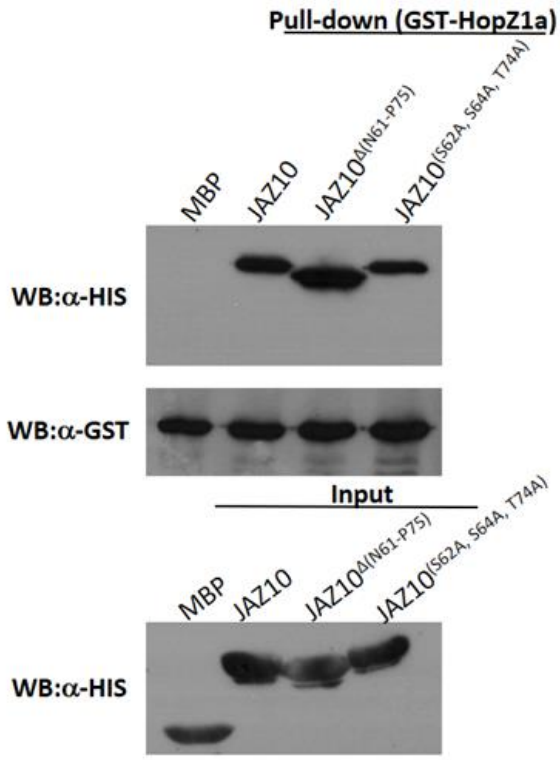


Figure III. HopZ1a interacts with JAZ10, JAZ10$^{(S62A, S64A, T74A)}$ and JAZ10$^{\Delta(N61-P75)}$ *in vitro*. In *E.coli* GST-HopZ1a was over-expressed and purified from whole cell lysate using glutathione resins and then incubated with an equal amount of whole cell lysate of *E. coli over*-expressing MBP-JAZ10-HIS, MBP-JAZ10$^{(S62A, S64A, T74A)}$-HIS, MBP-JAZ10$^{\Delta(N61-P75)}$-HIS, or pETMAL (empty vector). Western blotting indicated GST-HopZ1a-bound resin interaction with JAZ10 was not disrupted by mutated sites. (Experiment performed by Dr. Shushu Jiang)

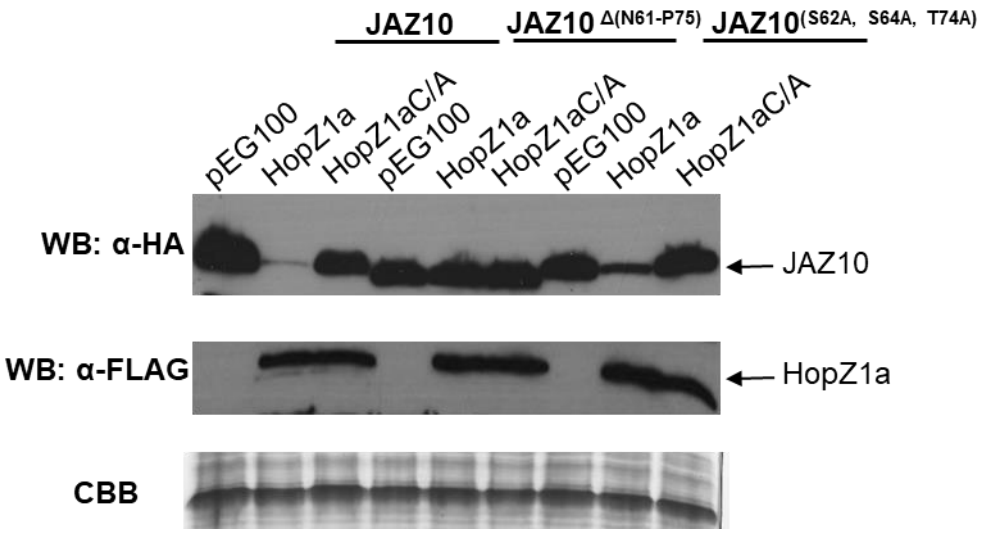


Figure IV. Reduction in degradation of JAZ10$^{(S62A, S64A, T74A)}$ and JAZ10$^{\Delta(N61-P75)}$. *Nicotiana benthamiana* was used to transiently co-express JAZ10-HA, JAZ10$^{(S62A, S64A, T74A)}$-HA, JAZ10$^{\Delta(N61-P75)}$-HA and 3x-FLAG-HopZ1a or 3x-FLAG-HopZ1aC/A. Samples were collected at 20 hpi and, using western blotting, a significant reduction of JAZ10 protein level in *N. benthamiana* leaves co-expressing HopZ1a, compared to leaves expressing the HopZ1aC/A or infiltrated with *Agrobacterium* carrying the empty vector was observed. Moreover, JAZ10$^{(S62A, S64A, T74A)}$ protein level was reduced in *N. benthamiana* leaves co-expressing HopZ1a, compared to leaves expressing the HopZ1aC/A or infiltrated with *Agrobacterium* carrying the empty vector. Finally, there was no visible reduction in the protein level of JAZ10 $^{\Delta(N61-P75)}$ when co-expressed with HopZ1a, HopZ1aC/A or empty vector. These results suggest that HopZ1a induces the degradation of JAZ10 and the acetylation of JAZ10 is required for degradation (Experiment performed by Dr. Shushu Jiang).

MATERIALS AND METHODS

Plant growth conditions

Arabidopsis thaliana seeds were sown in soil and vernalized at 4°C for 3 days. The plants were then grown in a conditioned growth room at 22°C with a 12/12 light/dark regime. *Nicotiana benthamiana* plants were geminated and grown in a conditioned growth room at 22°C with a 12/12 light/dark regime.

Transgenic plant generation

A single *Agrobacterium* colony was selected and grown in 5 mL of Luria-Bertani (LB) medium (BD Inc., United States) for 24-48 hours at 28°C. After growth, 250 µL of the bacterial suspension was inoculated into 250 mL of LB medium (BD Inc., United States) and grown out for 12 hours at 28°C until cell density measured OD_{600} = 1.0. Cells were collected by centrifugations at 6,000 x *g* for five minutes at 4°C. Cells were resuspended in infiltration media (1/2 MS salt, 5% sucrose, and 0.03% Silwet-77, pH=5.7) at OD_{600} = 0.8. Next, the floral dip method was used for *Arabidopsis* transformation (Clough and Bent, 1998). Plants with numerous immature floral buds and few siliques were inoculated by immersing the immature floral buds into the *Agrobacterium* solution for 10 minutes, then they were laid on the side of the pot in a flat tray with paper towels moistened with water. To keep humidity high, plastic wrap was also sprayed with water and placed on top of the plants as a cover. The plants were covered and left in the dark for 24 hours. The following day, the plants were sprayed with water to rinse off the silwet-77, then the plants were placed upright in the trays.

After collecting the seeds from these plants, they were grown out and selected using Bayer's Basta (phosphinothricin glufosinate) herbicide to check for plants carrying the transgene. Total protein was extracted from the plants to test for protein production using Western blotting (see below for details). Dr. Shushu Jiang generated *3xHA-JAZ10, 3xHA-JAZ10*$^{(S62A, S64A, T74A)}$ and *3xHA-JAZ10*$^{\Delta(N61-P75)}$ cloned under CaMV 35S promoter (Kay et al., 1987) into binary vector pJYP003 (Yang et al., 2012) then transformed *Agrobacterium* strain GV3101 (pMP90) (Holsters et al., 1980).

JAZ degradation in Arabidopsis

Five-week-old *Arabidopsis* plants expressing *35S-3xHA-JAZ10, 35S-3xHA-JAZ10*$^{(S62A, S64A, T74A)}$ *and 35S-3xHA-JAZ10*$^{\Delta(N61-P75)}$ were hand infiltrated with bacterial suspensions of *Pto*DC3000 (Cuppels, 1986) or *Pto*DC3118 (Moore et al., 1989) carrying empty vector pUCP18 (Schweizer, 1991), pUCP18::*HopZ1a-HA*, pUCP18::*HopZ1aC/A*-HA using OD_{600} = 0.2 (approximately 2 x 10^8 cfu/mL colony forming units). Bacterial strains used are from previous work published by our lab (Jiang et al., 2013). Eight hours post inoculation total proteins were extracted in 150 µL of 2xLaemmli buffer (Laemmli, 1970). Samples were boiled for 5 minutes then centrifuged at 15000g for 5 minutes. JAZ abundance was analyzed by Western blotting (see below for details). *Pseudomonas syringae* was grown on King's B medium (King et al., 1954) as previously described by Morgan et al. (2010).

In planta HopZ1a Interaction with JAZ10

To test for *in vivo* protein interaction, five-week-old *Nicotiana benthamiana* were co-infiltrated with *Agrobacterium* strain GV3101 (pMP90) carrying: pEG100::3xFLAG-HopZ1aC/A (Shushu Jiang, 2013) with pJYP003::3xHA-JAZ10, pJYP003::3xHA-JAZ10$^{(S62A,\ S64A,\ T74A)}$ and pJYP003::JAZ10$^{\Delta(N61\text{-}P75)}$ (constructs generated by Dr. Shushu Jiang) and pEG101::*RCN1*-YFP-HA as negative control (kindly provided by Dr. Tung Kuan). An *Agrobacterium* colony was grown into 5 mL Luria-Bertani (LB) medium (BD Inc., United States) and grown at 28°C for 24-48 hours. From this culture 100 µL were transferred into 10 mL of LB medium with 200 µL 0.5M MES (pH= 5.7), 4µL of 100mM acetosyringone, and cells were induced at 28°C for no more than 16 hours. Following induction, the cells were collected and resuspended in infiltration buffer (10mM $MgCl_2$, 10mM MES, 0.15mM acetosyringone). Cell densities for HopZ1aC/A, JAZ, and RCN1 were adjusted to OD_{600} of 1.5 while P19 (viral RNA silencing suppressor) cells were adjusted to OD_{600} of 1.0. Equal volumes of *Agrobacterium* suspensions HopZ1aC/A, JAZ, RCN1 or P19 were mixed and hand infiltrated into 4-week-old *N. benthamiana* leaves. 20 hours post infiltration, tissues were collected and ground in protein extraction buffer (25mM Tris-HCl pH=8.0, 1mM EDTA, 150mM NaCl, 5mM DTT, .1% NP-40, 10%

glycerol, 1x protease inhibitor) then incubated on anti-FLAG agarose beads (Sigma-Aldrich Inc., United States) for 1 hour at 4°C on a rotator. Post incubation on the beads, samples were washed with 1 mL of protein extraction buffer and after each wash some resin was saved (output). After three washes, the resin was mixed with 2xLaemmli buffer (Laemmli, 1970) boiled for 5 minutes. Proteins were detected by Western blotting (see below for details) using anti-FLAG or anti-HA antibodies.

Arabidopsis T-DNA insertion mutant genotyping

Genotyping of Arabidopsis T-DNA insertion SALK lines were followed according to the online website “T-DNA Primer Design” (http://signal.salk.edu/tdnaprimers.2.html) provided by Salk Institute Genomic Analysis Laboratory. Briefly, LP (left genomic primer) and RP (right genomic primer) specific to individual SALK line, and the LBb1.3 (left border primer of the T-DNA insertion, 5’-ATTTTGCCGATTTCGGAAC-3’) were used for *zar1-1* mutant (SALK_013297) genotyping PCR. Wild-type plants with no insertion were expected to have PCR amplicons about 1156 bps (from LP to RP). Homozygous mutants with insertions in both chromosomes were expected to have amplicons size of 510-810 (410+N bps from RP to insertion site 300+N bases, plus 110 bases from LBb1.3 to the left border of the vector). Heterozygous mutants with insertion into only one chromosome were expected to have both PCR bands. Note that the *jaz10* mutant used in this study was a SAIL line (SAIL_92_D08)

requiring LB3 (5'-TAGCATCTGAATTTCATAACCAATCTC-GATACAC-3') as the left border primer of the T-DNA insertion for genotyping. Wild-type plants with no insertion were expected to have PCR amplicons about 1086 bp (from LP to RP) Homozygous mutants with insertions in both chromosomes were expected to have amplicons size of 549-849 bp. *Arabidopsis* ubiquitin 10 *(UBQ10)*, was used as internal controls for genomic DNA PCR. UBQ10 forward primer (5'- AAATC TCGTCTCTGTT ATGCTTAAGAAG-3') and UBQ10 reverse primer (5'- AAAGA GATAACAGGAACGGA AACATAGT-3'). Professor Sheng Yang He at Michigan State University kindly provided the *jaz10* homozygous line (SAIL_92_D08), used for the *jaz10/zar1-1* (double null) background.

Reverse transcription (RT)-PCR

Total RNA was extracted from *Arabidopsis* tissues by grinding approximately 0.1g of leaf tissue in liquid nitrogen followed by re-suspension in 1 mL TRIzol (Ambion Inc., United States). 200 µL of chloroform was used to separate the solid and aqueous phases, followed by centrifugation at 15,000 x *g* for 15 minutes and 4°C. The aqueous phase (containing RNA) was precipitated for 20min at -20C in 1 mL of isopropanol. Pellets were centrifuged (at 15,000 x *g*) and washed two times with 75% molecular grade ethanol, then air dried in the chemical hood before re-suspension in sterile water. RNA yield and quality were measured using the NanoDrop 2000 Spectrophotometer (Thermo Scientific Inc., United States). 1µg of total RNA was subjected to DNAse treatment reverse

transcribed by RevertAid Reverse Transcriptase with RiboLock RNase Inhibitor at 42°C for one hour using oligo-dT as a primer (Kit from Thermo Scientific Inc., United States). The synthesized cDNA then served as templates for PCR using *Arabidopsis JAZ10* (AT5G13220) gene-specific primers. The primers were designed by Dr. Shushu Jiang to amplify a 399 bp PCR product in *JAZ10* cDNA. *JAZ10* forward (5'-TCGGC TAAATCTCGTTCGGTT-3') and *JAZ10* reverse (5'-AGAGCGGCCGCGGCCGATGTCG-3').

Hypersensitive response assay

Five-week-old *Arabidopsis* Col-0, *zar1-1*, *jaz10/zar1-1* lines #5 and #8 (F3 generation) were hand infiltrated with bacterial suspensions of *Pto*DC3118 carrying pUCP18::*hopZ1a-HA*, pUCP18::*hopZ1aC/A-HA* (under native promoter), pUCP18 empty vector using OD_{600} = 0.2 (approximately 2 x 10^8 cfu/mL) or infiltration buffer (10mM $MgSO_4$). 24 hours post inoculation hypersensitive response was observed.

In planta bacterial growth assay

Following a previously published *Pseudomonas syringae* infection assay (Yao et al., 2013), five-week-old *Arabidopsis 35S-3xHA-JAZ10* (#4 in Western blot Figure 5)*, 35S-3xHA-JAZ10*$^{(S62A,\ S64A,\ T74)A}$ (#3 in Western blot Figure 5) *and 35S-3xHA- JAZ10*$^{\Delta(N61\text{-}P75)}$ (#4 in Western blot Figure 5) in *jaz10/zar1-1* background (line 5) were hand infiltrated with bacterial suspensions of

*Pto*DC3000 (OD_{600}= 0.0001 approximately 1 x 10^5 cfu/mL) or *Pto*DC3118 carrying pUCP18 empty vector, pUCP18::*HopZ1a-HA*, pUCP18::*HopZ1aC/A-HA* (OD_{600}= 0.002 approximately 1 x 10^6 cfu/mL). The inoculated plants were covered to maintain 90% humidity. Bacterial populations were determined 3 days post inoculation as colony forming units per cm^2 using a previously described procedure (Morgan et al., 2010) The average colony forming units per square centimeter (cfu/cm^2) and standard deviations (as error bars) are presented. Different letters at the top of the bars represent data with statistically significant differences (two tailed t-test p<0.01).

Western blotting

For the Western blots, total proteins from plant tissues or Co-IP were prepared as described: 2xLaemmli buffer (Laemmli, 1970) was added to all protein extracts and samples were boiled for 5 min before separation by 12% polyacrylamide gels via SDS-PAGE. Gels were transferred to PVDF membrane paper and blocked with blocking buffer (1xTBST, pH=7.4, containing 5% w/v non-fat milk) 1xTBS-T buffer (0.15M Sodium Chloride, 0.02M Tris-base (pH=7.6) and 0.2% Tween-20) at room temperature for 15 minutes, followed by incubation with the appropriate primary antibody either, anti-HA (Roche Inc., United States) (1:1000 dilution) or anti-FLAG antibody (Clontech Inc., United States) (1:1000 dilution). Membranes were then washed with TBS-T 3 times for 5 minutes each wash. Membranes that were incubated with anti-HA were then incubated with

secondary goat anti-rat IgG horseradish peroxidase (HRP)-conjugated antibody (Santa Cruz Biotechnology Inc., United States) (1:5000 dilution) at room temperature for 1 hour. Membranes incubated with anti-FLAG antibody were then incubated with secondary goat-anti-mouse IgG horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotechnology Inc., United States) (1:5000 dilution) at room temperature for 1 hour. Signals for antibody-bound proteins were detected with SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific Inc., United States).

RESULTS

HopZ1a induces degradation of JAZ10 but not JAZ10$^{(S62A, S64A, T74A)}$ or JAZ10$^{\Delta(N61-P75)}$ in Arabidopsis during P. syringae infection

Dr. Shushu Jiang's results confirmed decreased HopZ1a degradation of JAZ10$^{(S62A, S64A, T74A)}$ and JAZ10$^{\Delta(N61-P75)}$ using *N. benthamiana.* Expression of HopZ1a in *N. benthamiana* eventually triggers HR due to the presence of resistance protein ZAR1, possibly interfering with the results. In order to consolidate the data, I transformed *Arabidopsis* in *zar1-1* background to ensure no HR will compromise the data. *Agrobacterium* carrying *3xHA-JAZ10, 3xHA-JAZ10$^{(S62A, S64A, T74A)}$,* and *3xHA- JAZ10$^{\Delta(N61-P75)}$* was used to transform *Arabidopsis*. I selected transgenic homozygous *Arabidopsis* lines that strongly expressed 3xHA-JAZ10, 3xHA-JAZ10$^{(S62A, S64A, T74A)}$, and 3xHA- JAZ10$^{\Delta(N61-P75)}$

(Figure 1). I hand infiltrated 3xHA-JAZ10 (#5 from Western blot), 3xHA-JAZ10$^{(S62A, S64A, T74A)}$ (#7 from Western blot), and 3xHA- JAZ10$^{\Delta(N61\text{-}P75)}$ (#2 from Western blot) with four bacterial *P. syringae* pv. *tomato* suspensions: *Pto*DC3000, *Pto*DC3118 expressing HopZ1a-HA or HopZ1aC/A-HA, and *Pto*DC3118 (empty vector). Eight hours post inoculation I collected the samples and analyzed the protein abundance using Western blotting. *Pto*DC3000 is well-known to induce JAZ degradation through the production of coronatine (Katsir et al., 2008b), so it served as a control for JAZ degradation. *Pto*DC3118 is a coronatine mutant; therefore, JAZ10 degradation would be due to HopZ1a. The mutant *Pto*DC3118 expressing HopZ1a-HA no longer degrades 3xHA-JAZ10$^{(S62A, S64A, T74A)}$, and 3xHA- JAZ10$^{\Delta(N61\text{-}P75)}$; therefore, degradation of JAZ10 is a consequence of HopZ1a acetylation **(Figure 2)**.

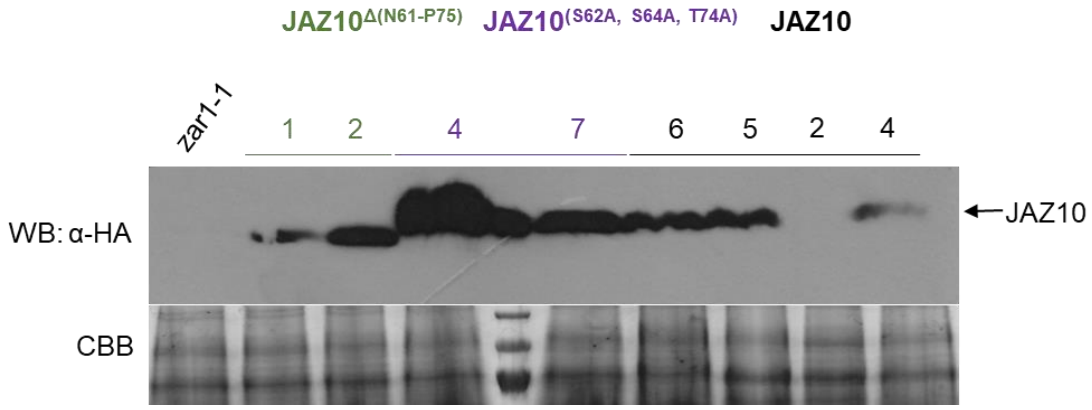


Figure 1. Transgenic *Arabidopsis* expressing 3xHA-JAZ10, 3xHA-JAZ10$^{(S62A, S64A, T74A)}$, and 3xHA- JAZ10$^{\Delta(N61-P75)}$ in *zar1-1* mutant background. Homozygous transgenic plants were selected for Western blotting detection of JAZ expression using anti-HA antibody.

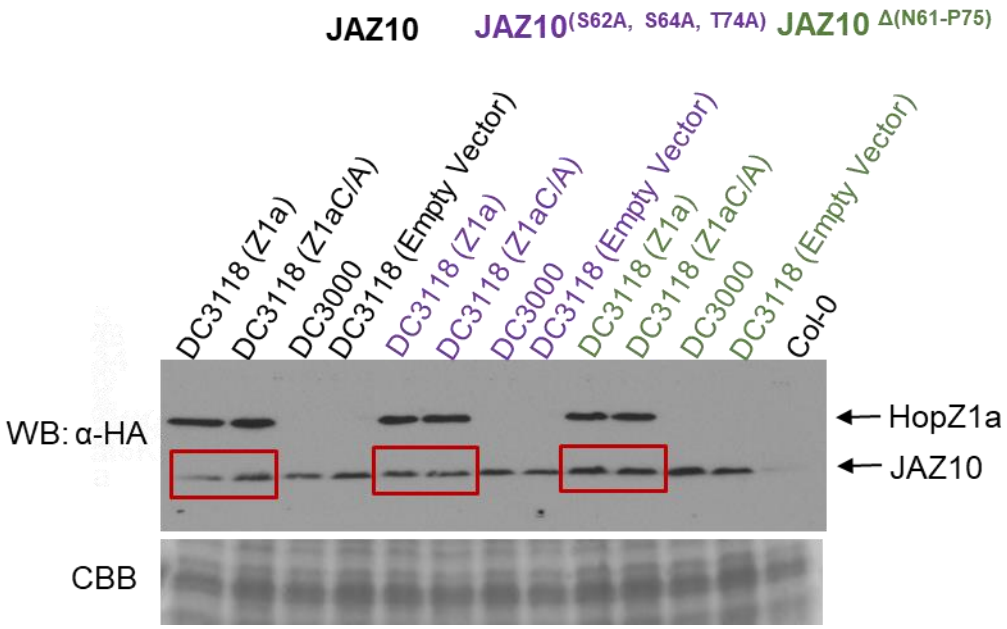


Figure 2. HopZ1a induces degradation of JAZ10 but not JAZ10$^{(S62A, S64A, T74A)}$ or JAZ10$^{\Delta(N61-P75)}$ during *P. syringae* infection. Transgenic *Arabidopsis* expressing 3xHA-JAZ10, 3xHA-JAZ10$^{(S62A, S64A, T74A)}$, and 3xHA- JAZ10$^{\Delta(N61-P75)}$ were hand infiltrated with *Pto*DC3000, *Pto*DC3118 expressing HopZ1a-HA or HopZ1aC/A-HA from their native promoter, and *Pto*DC3118 (empty vector) OD_{600}=0.2 (approximately 2 x 10^8 cfu/mL). Eight hours post inoculation I collected the samples and analyzed the protein abundance using Western blotting. Gel stained with Coomassie brilliant blue (CBB) served as loading control.

HopZ1a interaction with JAZ10 cannot be detected in vivo

Dr. Shushu Jiang confirmed interaction between HopZ1a and JAZ10 was not disrupted by the acetylation site mutations using *in vitro* pull-down (Figure IV). In order to prove *in vivo* interaction, I carried out Co-immunoprecipitation (Co-IP) using FLAG-tagged HopZ1aC/A and HA-tagged JAZ10, JAZ10$^{(S62A,\ S64A,\ T74A)}$, and JAZ10$^{\Delta(N61\text{-}P75)}$ were co-expressed in *N. benthamiana* using *Agrobacterium* mediated transient expression. I used HopZ1aC/A because HopZ1a will elicit hypersensitive response in *N. benthamiana* compromising the result. Total proteins were extracted from infiltrated leaves and incubated with anti-FLAG agarose beads. I could not detect any interaction using Western blotting despite having Dr. Jiang's *in vitro* result confirming interaction. JAZ6 was previously shown to interact with HopZ1a *in vitro*, but it also did not interact with HopZ1aC/A *in vivo* **(Figure 3)**. RCN1 (roots curl in NPA) is an *Arabidopsis* PP2A subunit A isoform (Farkas et al., 2007) and it served as a negative control for interaction with HopZ1aC/A. RCN1-YFP-HA was kindly provided by Dr. Tung Kuan.

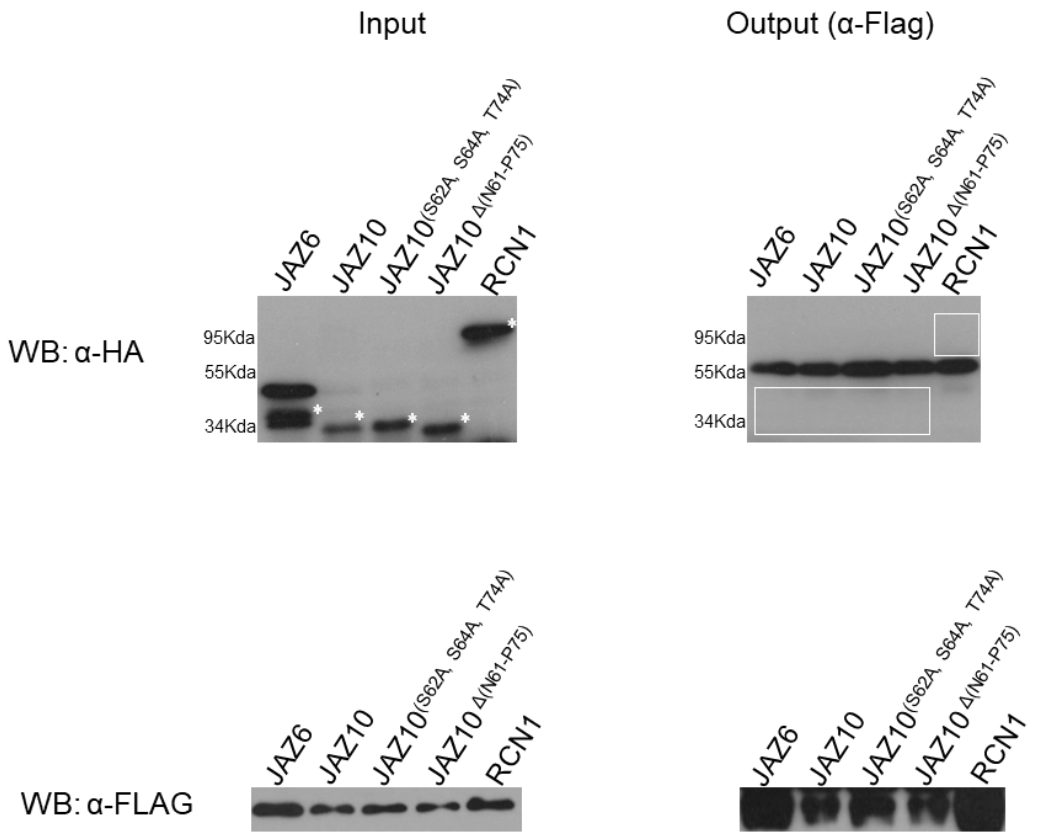
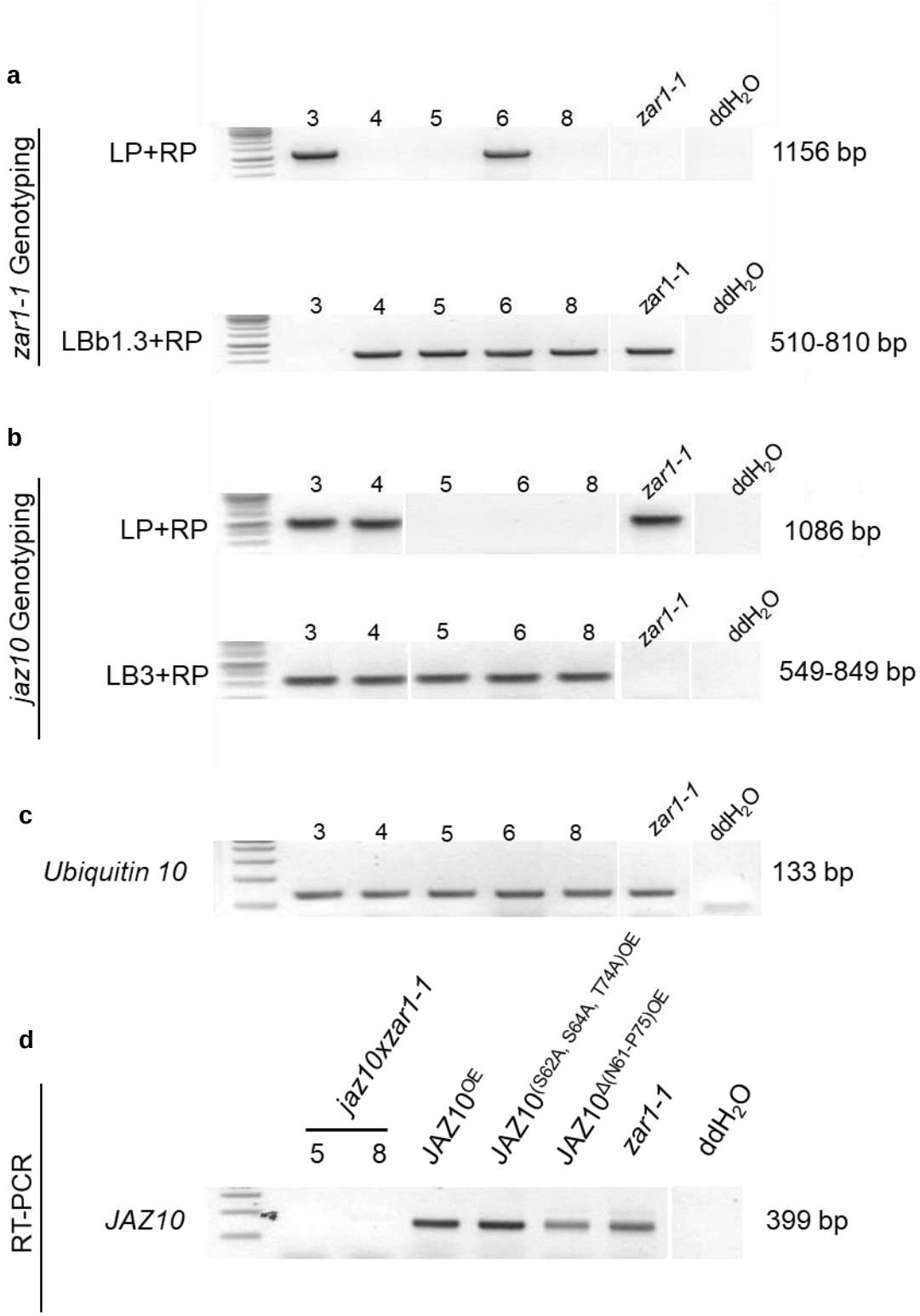


Figure 3. HopZ1a interaction with JAZs cannot be detected *in vivo*.
3xHA-JAZ10, 3xHA-JAZ10$^{(S62A, S64A, T74A)}$, and 3xHA- JAZ10$^{\Delta(N61-P75)}$ were co-expressed with HopZ1aC/A-3xHA from their native promoter, and the total protein extracts were subjected to Co-IP assay with anti-FLAG agarose beads. Immunoprecpitates were detected using anti-FLAG and anti-HA by Western blotting. RCN1-YFP-HA was used as negative control.

Generation of jaz10/zar1-1 double null Arabidopsis

To study whether reduction in acetylation and degradation causes JAZ10$^{(S62A, S64A, T74A)}$, and JAZ10$^{\Delta(N61\text{-}P75)}$ plants to be less susceptible to infection by HopZ1a, I firstly needed *Arabidopsis* without endogenous JAZ10. I generated *Arabidopsis* plants null for JAZ10 and ZAR1 by crossing *zar1-1* T-DNA insertion line (SALK _013297) (the pistil donor) with *jaz10* T-DNA insertion line (SAIL_92_D08) (the pollen donor) and obtained homozygous generation confirmed by genotyping PCR. Left border primer (LP) and right border primer (RP) for *ZAR1* and *JAZ10* showed no PCR product for line 5 and line 8 **(Figure 4a** and **Figure 4b)**, indicating no presence of wild-type genes. I pursued lines 5 and 8 using genotyping PCR with left border of the T-DNA insertion (LBb1.3) and right border primer (RP), and both lines showed a PCR product at 510-810 bp, confirming presence of T-DNA insertion in *zar1-1* **(Figure 4a)**. I performed genotyping PCR with left border of the T-DNA insertion (LB3) and right border primer (RP) on lines 5 and 8 and both lines showed a PCR product at 549-849 bp, confirming presence of T-DNA insertion in *jaz10* **(Figure 4b)**. Reverse transcription (RT)-PCR analysis for measuring *JAZ10* gene expression in *zar1-1/jaz10* lines 5 and 8 mutants showed no transcript for JAZ10 in line 5 but there was a very faint band in line 8 **(Figure 4c)**; furthermore, overexpression lines JAZ10, JAZ10$^{(S62A, S64A, T74A)}$, and JAZ10$^{\Delta(N61\text{-}P75)}$ (Figure 1) in *zar1-1* background served as positive controls. Ubiquitin 10 served as internal control for genotyping **(Figure 4d)**. Finally, I hand infiltrated *Arabidopsis* Col-0, *jaz10/zar1-1* line 5,

jaz10/zar1-1 line 8 and *zar1-1* with *Pto*DC3118 carrying HopZ1a, *Pto*DC3118 carrying HopZ1aC/A, *Pto*DC3118 carrying empty vector, or infiltration buffer ($MgSO_4$) and I only detected hypersensitive response in Col-0 infiltrated with *Pto*DC3118 carrying HopZ1a, confirming *jaz10/zar1-1* line 5*, jaz10/zar1-1* line 8 do not express wild type resistance protein ZAR1 **(Figure 4e)**.


a
zar1-1 Genotyping
LP+RP
3
4
5
6
8
zar1-1
ddH2O
1156 bp
LBb1.3+RP
510-810 bp
b
jaz10 Genotyping
LP+RP
1086 bp
LB3+RP
549-849 bp
c
Ubiquitin 10
133 bp
d
RT-PCR
jaz10xzar1-1
JAZ10OE
JAZ10(S62A, S64A, T74A)OE
JAZ10Δ(N61-P75)OE
JAZ10
399 bp

e

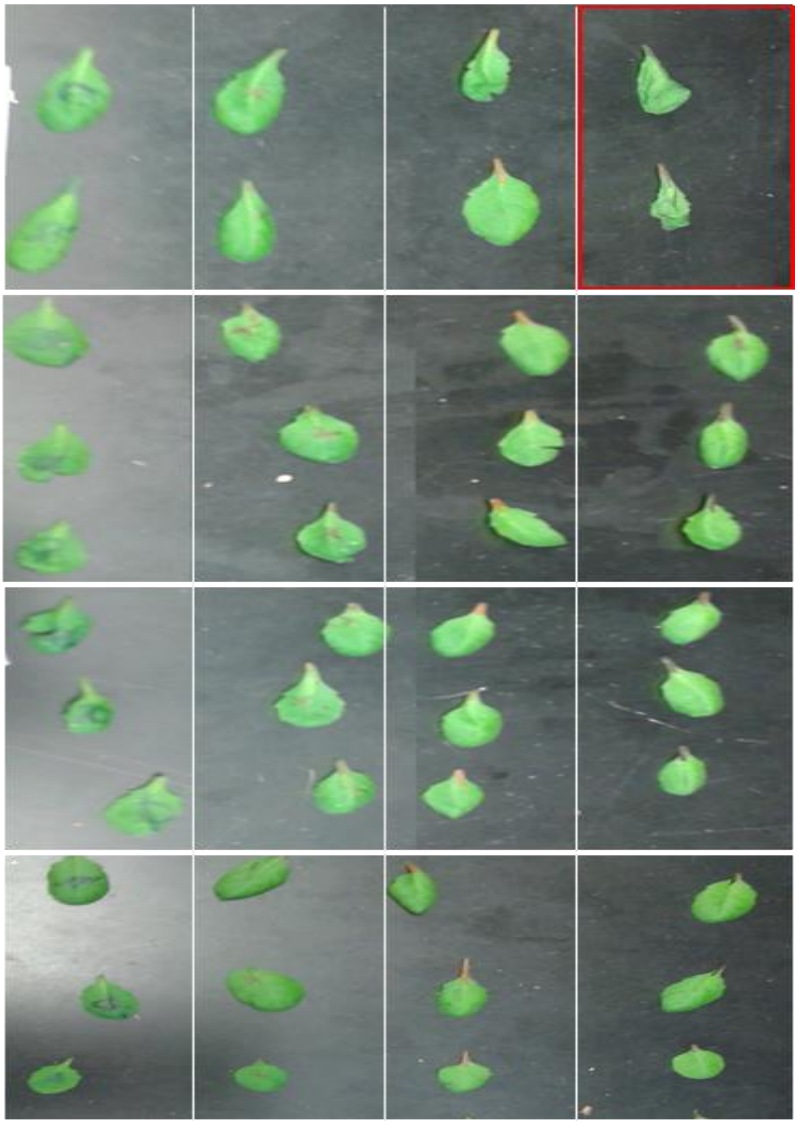

DC3118 (Z1a)
DC3118 (Z1aC/A)
DC3118 (EV)
Infiltration buffer
Col-0
jaz10/zar1-1 Line 5
jaz10/zar1-1 Line 8
zar1-1

Figure 4. Screening of *jaz10/zar1-1* double null crossed *Arabidopsis* lines 5 and 8. PCR based genotyping of **(a)** *zar1-1* T-DNA insertion mutants, **(b)** *jaz10* T-DNA insertion mutants, where LP and RP (left and right are genomic primers), together with LBb1.3 and LB3 (left border of the T-DNA insertion) were used for genotyping (see Materials and Methods). **(c)** RT-PCR analyses of cDNA from lines 5 and 8 were performed using primers indicated in Materials and Methods showing lack of *JAZ10* mRNA. *Arabidopsis* lines over-expressing JAZ10, JAZ10$^{(S62A, S64A, T74A)}$, and JAZ10$^{\Delta(N61\text{-}P75)}$ were used as positive control as well as *zar1-1* and Col-0 endogenously expressing JAZ10. **(d)** Ubiquitin 10 was used as an internal control. **(e)** *P. syringae* assay showing lack of hypersensitive response in *jaz10/zar1-1* line 5 and line 8 when infected with *Pto*DC3118 carrying HopZ1a compared to Col-0 expressing wild-type ZAR1 which shows hypersensitive response.

Generation of JAZ10, JAZ10$^{(S62A, S64A, T74A)}$, and JAZ10$^{\Delta(N61-P75)}$ in jaz10/zar1-1 background

In order to elucidate if JAZ10$^{(S62A, S64A, T74A)}$, and JAZ10$^{\Delta(N61-P75)}$ plants are less susceptible to infection, I transformed *Arabidopsis jaz10/zar1-1* line 5 and line 8 generated in Figure 4 with *Agrobacterium* carrying *35S-3xHA-JAZ10, 35S-3xHA-JAZ10$^{(S62A, S64A, T74A)}$*, and *35S-3xHA- JAZ10$^{\Delta(N61-P75)}$*. Multiple independent homozygous lines of transgenic *Arabidopsis* plants can strongly express protein **(Figure 5).** I used *Arabidopsis jaz10/zar1-1* line 5 strongly expressing 3xHA-JAZ10 (#4 in Western blot Figure 5), 3xHA-JAZ10$^{(S62A, S64A, T74)}$ (#3 in Western blot Figure 5) and 3xHA-JAZ10$^{\Delta(N61-P75)}$ (#4 in Western blot Figure 5) plants to perform bacterial growth assay. I hand infiltrated all three transgenic lines including *jaz10/zar1-1* line 5 with three bacterial *P. syringae* pv. *tomato* suspensions: *Pto*DC3000, *Pto*DC3118 carrying *hopZ1a-3xHA* or, *Pto*DC3118 (empty vector). I observed significantly less bacterial growth in plants expressing 35S-3xHA-JAZ10$^{(S62A, S64A, T74A)}$, and 35S-3xHA- JAZ10$^{\Delta(N61-P75)}$ than plants expressing 35S-3xHA-JAZ10. *jaz10/zar1-1* plants infected with *Pto*DC3118 carrying *hopZ1a-3xHA* exhibited more bacterial growth when infected with *Pto*DC3118 carrying empty vector **(Figure 6)**.

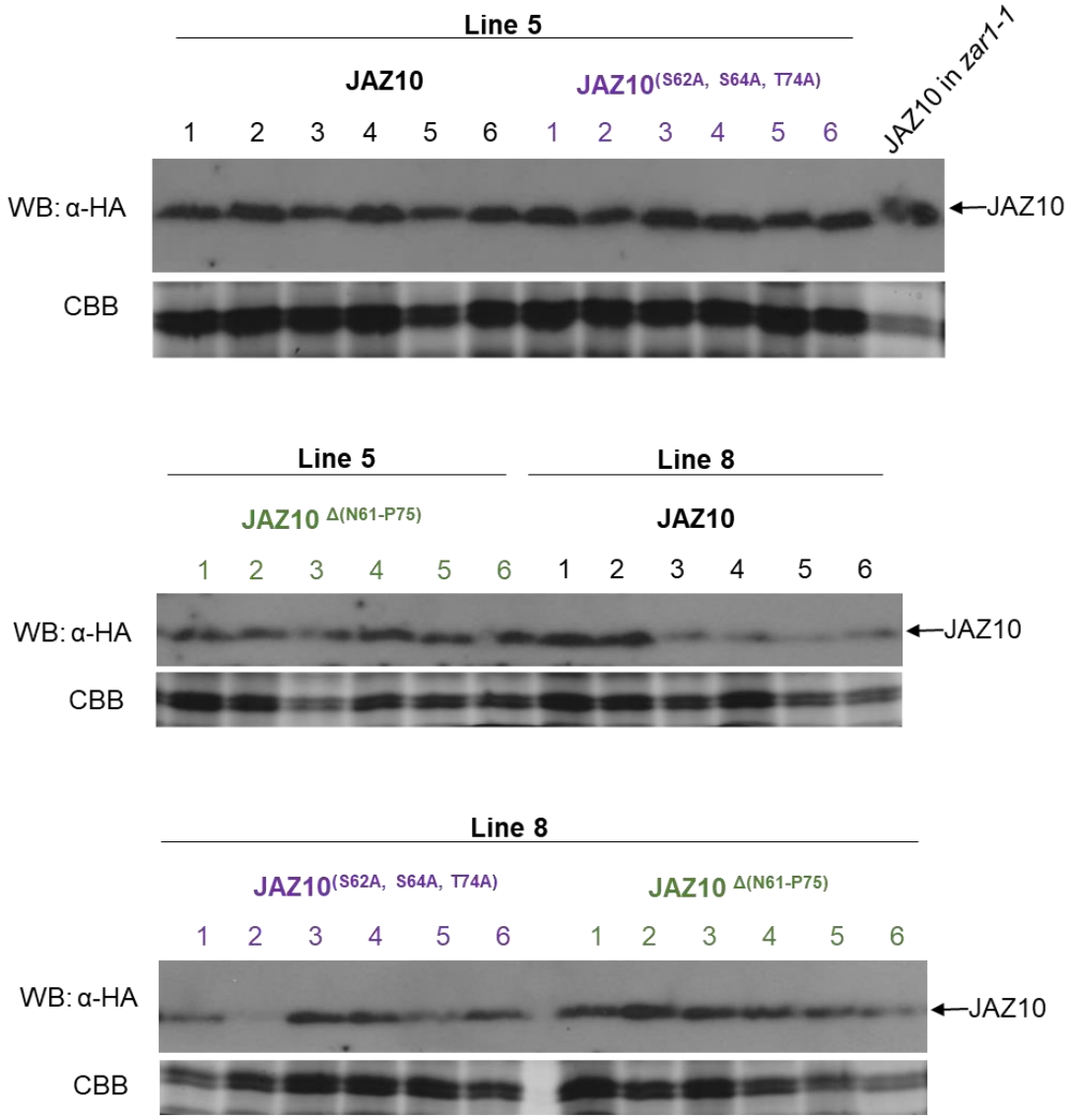


Figure 5. Transgenic *Arabidopsis* expressing 3xHA-JAZ10, 3xHA-JAZ10$^{(S62A, S64A, T74A)}$, and 3xHA- JAZ10$^{\Delta(N61-P75)}$ in *jaz10/zar1-1* mutant background. Homozygous transgenic plants were selected for Western blotting detection of JAZ expression using anti-HA antibody. Gels stained with Coomassie brilliant blue (CBB) served as loading control.

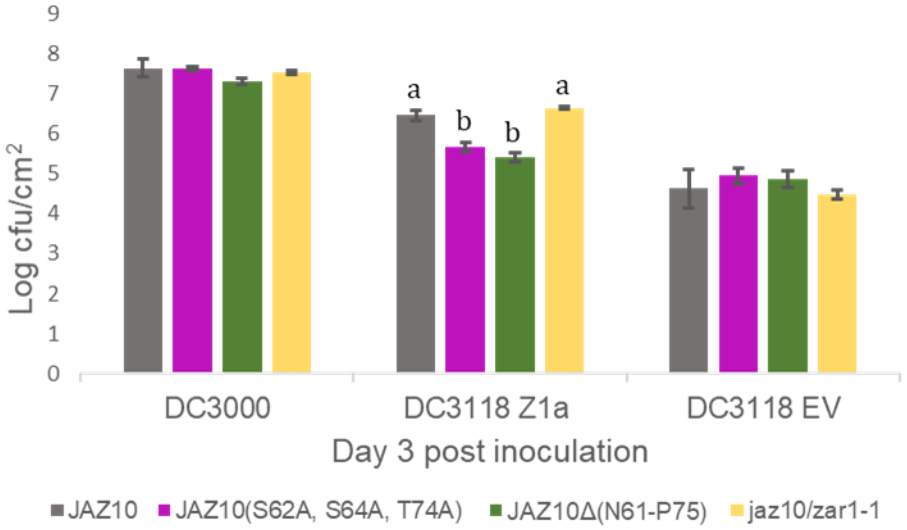


Figure 6. Transgenic *Arabidopsis* plants expressing 3xHA-JAZ10$^{(S62A, S64A, T74A)}$ and 3xHA- JAZ10$^{\Delta(N61\text{-}P75)}$ are less susceptible to infection by DC3118 carrying HopZ1a-HA from its native promoter than plants expressing 3xHA-JAZ10. *Arabidopsis jaz10/zar1-1* line 5 over-expressing 3xHA-JAZ10 (#4 in Western blot in Figure 5), 3xHA-JAZ10$^{(S62A, S64A, T74A)}$ (#3 in Western blot Figure 5) and 3xHA- JAZ10$^{\Delta(N61\text{-}P75)}$ (#4 in Western blot Figure 5) were hand-infiltrated with *Pto*DC3000 (OD_{600}= 0.0001 approximately 1 x 10^5 cfu/mL), *Pto*DC3118 carrying pUCP18::*hopZ1a-HA*, or *Pto*DC3118 carrying pUCP18 empty vector (OD_{600}= 0.002 approximately 1 x 10^6 cfu/mL). Bacterial populations were determined at 3 days post inoculation. The average colony forming units per square centimeter (cfu/cm^2) and standard deviations (as error bars) are presented. Different letters at the top of the bars represent data with statistically significant differences (two tailed t-test p<0.01).

DISCUSSION

Type III secreted effector HopZ1a belongs to the YopJ family of effectors whose members consist of plant and animal pathogens (Gupta, 2000). While HopZ1a's acetyltransferase function and ability to target JAZs, leading to their degradation, has been established, there remained a missing link between these two observations. Using *P. syringae* infection assay I observed hindrance of HopZ1a mediated degradation of JAZ10$^{(S62A,\ S64A,\ T74A)}$ and JAZ10$^{\Delta(N61\text{-}P75)}$. Surprisingly, *Pto*DC3000 carrying coronatine did not degrade JAZ10 when it was intended to serve as positive control meaning coronatine may require a longer time point to degrade JAZ10. To elucidate whether JAZ10$^{(S62A,\ S64A,\ T74A)}$ and JAZ10$^{\Delta(N61\text{-}P75)}$ plants are less susceptible to infection by HopZ1a, I performed a bacterial growth assay which indicated they are in fact significantly less susceptible to infection than JAZ10 plants. While Dr. Shushu Jiang's *in vitro* data does not show abolishment in HopZ1a acetylation of JAZ10$^{(S62A,\ S64A,\ T74A)}$ and JAZ10$^{\Delta(N61\text{-}P75)}$, there are many other potential acetylation sites but they are present in HopZ1aC/A, indicating their acetylation may not be associated with the activity we are interested in. Furthermore, *jaz10/zar1-1* plants infected with *Pto*DC3118 carrying HopZ1a were more susceptible than plants infected with *Pto*DC3118 carrying empty vector, indicating there may be another JAZ protein being targeted and degraded in the absence of JAZ10 due to functional redundancy (Chini et al., 2007; Thines et al., 2007). HopZ1a interacts with and acetylates multiple JAZs (Jiang et al., 2013). Transcriptomic analysis might help

us understand which JAZ proteins are upregulated in the absence of JAZ10, JAZ10$^{(S62A, S64A, T74A)}$, and JAZ10$^{\Delta(N61\text{-}P75)}$ in *jaz10/zar1-1* during *P. syringae* infection. This however is not an insignificant task due to the rapid initial stress response post infection, it is difficult to determine exactly when pathogen-induced expression begins, but it is likely that pathogen-derived signals, such as COR or HopZ1a, continue to stimulate JA signaling after the initial stress signal subsides (Demianski et al., 2012).

HopZ1a interaction with JAZ10, JAZ10$^{(S62A, S64A, T74A)}$, and JAZ10$^{\Delta(N61\text{-}P75)}$ was confirmed *in vitro* by Dr. Shushu Jiang; however, I could not prove this *in vivo.* This could be due to several factors, 1) JAZs role in phytohormones suggesting the interaction is easily disrupted 2) It is also likely this association occurs quickly in the plant; therefore, *in vitro* interaction is more adept in detecting the interaction. Finally, 3) JAZs are nuclear proteins (Chini et al., 2007; Thines et al., 2007), whereas HopZ1a is a membrane-associated protein (Lewis et al., 2008); however, it has been shown that a sub-pool of HopZ1a might go to nucleus (Zhou et al., 2011; Jiang et al., 2013). Nonetheless, my *in vivo* Co-IP experiments were performed using *Agrobacterium* transient expression in *N. benthamiana* which may be too artificial of a system to capture the nuanced localization. Interestingly, neither HopX1 or MiSSP7 were shown to interact with their JAZ targets using co-expression *in planta* and Co-immunoprecipitation. Perhaps utilizing techniques such as transient expression in *Arabidopsis* protoplast (Yoo et al., 2007), semi *in vitro* expression with one plant expressed

protein and one *E. coli* expressed protein, or TurboID (Branon et al., 2018) may be more suitable for capturing direct interactions with JAZs as microbial targets.

The redundant nature of JAZs poses a concern in the mind of researchers due to SA-JA antagonism. If one mutated JAZ shows a promising phenotype when challenged by a bacterium, any agriculturally relevant design leading to resistance to biotrophic pathogens must not also lead to penalties of enhanced susceptibility to necrotrophic pathogens (Wasternack, 2017). Gimenez-Ibanez and colleagues showed cell-specific expression of *JAZ2* at guard cells and the compromised function of JAZ2 is restricted to stomata of plants (Gimenez-Ibanez et al., 2017). Using this knowledge, the functional ortholog of the coronatine stomatal co-receptor AtJAZ2 in tomato (*SlJAZ2*) was edited using CRISPR/Cas9 to generate dominant JAZ2 repressors lacking the C-terminal Jas domain (*Sl*JAZ2Δjas) where stomatal reopening by coronatine was prevented and it also provided resistance to *Pto*DC3000 (Ortigosa et al., 2019). Importantly, resistance to the necrotrophic fungal pathogen *Botrytis cinereal* remained unaltered in *Sljaz2Δjas* plants (Ortigosa et al., 2019). Similar to coronatine, HopZ1a and AvrB are also able to inhibit stomatal defense and promote bacterial entry to apoplastic space (Ma et al., 2015; Zhou et al., 2015). The general strategy to uncouple SA-JA hormonal antagonism is promising especially since researchers have uncovered clues that guide us to focus on certain JAZs. For example, *JAZ10* was only moderately induced after herbivory; however, it was one of the most highly induced genes in response to *Pto*DC3000 (Demianski et al., 2012).

Thus, although both responses are mediated via JA signaling, there are also unique regulatory events governing the expression of specific *JAZ* genes in response to different stimuli (Demianski et al., 2012). These prospects might overcome the long-term drawbacks in manipulation of resistance against biotrophic and necrotrophic pathogens, and might ultimately lead to durable and sustainable broad-spectrum resistance in the field (Wasternack, 2017). More research is needed on JAZ10$^{(S62A,\ S64A,\ T74A)}$ and JAZ10$^{\Delta(N61\text{-}P75)}$ to understand these mutants' fitness perhaps when stressed or infected with a necrotroph; nonetheless, gaining insight into HopZ1a acetylation-mediated degradation of its targets is significant considering six other YopJ effectors (YopJ, AvrA, and VopA from the animal pathogens and HopZ3, PopP2, and AvrBsT from the plant pathogens) have been shown to acetylate their corresponding host targets (Mukherjee, 2006; Trosky et al., 2007; Jones et al., 2008; Lee et al., 2012; Cheong et al., 2014; Sarris et al., 2015; Ma and Ma, 2016b). In the arms race between pathogens and hosts, mechanistic knowledge of effectors may provide essential elements to generate host resistance in the future.

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Chapter 2

Optimization of Huanglongbing detection using *Candidatus* Liberibacter asiaticus and *Liberibacter crescens* Components

ABSTRACT

One of the main management strategies for HLB is the detection and removal of infected trees; however, the uneven distribution of the bacteria obscures accurate identification. Our lab pursued the use of *C*Las Sec-Delivered Effectors (SDEs) as biomarkers for detection of infected trees based on the premise that secreted proteins, with small molecular weight, might move more evenly within the phloem unlike the bacterial cells making them. In this chapter I discuss my contribution in confirming and optimizing the use of SDE1 (CLIBASLIA_05315) for direct tissue blot immunoassay (DTBIA) as well as generating *Liberibacter crescens* (*L. crescens)* cell and lipopolysaccharide (LPS) specific antibodies to serve as a cocktail primary antibody for enzyme-linked immunosorbent assay (ELISA). Furthermore, upon purification of *L. crescens* LPS, we had the structure analyzed by the Complex Carbohydrate Research Center (CCRC) to gain insight into the similarities and differences between the culturable and unculturable Liberibacters.

Our lab generated a list of predicted secreted effectors unique to *C*Las. From this list I confirmed that anti-SDE1 purified antibody is more efficient in detecting HLB infected plants compared to anti-SDE2 (CLIBASIA_03230) or anti-SDE1-peptide-2 (amino acids from Gly^{131} to Met^{150}). I confirmed that there is a

correlation between the strength of anti-SDE1 antibody detection and the infection stage by comparing symptomatic and asymptomatic trees. I confirmed the efficiency of anti-SDE1 antibody to detect infected tissue via Western blotting by testing PCR+ trees collected from the Contained Research Facility at UC Davis and a PCR+ tree collected from the Citrus Clonal Protection Program at UC Riverside. Upon the successful culturing of *L. crescens*, I generated cell and LPS antibody with the presumption their structures are similar to the unculturable *C*Las to further improve HLB detection. I sent *L. crescens* for LPS extraction and analysis at the CCRC then analyzed their results which indicated that there is 27OHC28:0, a very long chain fatty acid (VLCFA), present in *L. crescens* but the genes required for its synthesis are absent from all the pathogenic Liberibacters suggesting this maybe involved in the difficulty of culturing them. Finally, our collaborator's attempt to knockout the gene involved for synthesis of this VLCFA have failed likely due to the inability to grow *L. crescens* without this component. This data elucidates the importance of *L. crescens* as a tool to study *C*Las and HLB disease.

INTRODUCTION

Candidatus Liberibacter asiaticus (*C*Las) is the associated agent of the severe citrus disease Huanglongbing (HLB). HLB is the most destructive disease of citrus, having reached almost every major citrus producing region in the world. Symptoms include: yellowing of shoots, blotchy mottled leaves, malformed and

discolored fruit with bitter taste, premature fruit drop, root loss, and eventually tree death (J.M. Bové, 2006; Gottwald et al., 2007; Wang and Trivedi, 2013; Raithore et al., 2015). HLB has spread to all the citrus producing areas in the United States leading to the decrease in the production of oranges for processing from 7.98 to 2.22 billion tons (72.2% reduction) from 2007–2008 to 2017–2018 throughout the country (Dala-Paula et al., 2019). In Sao Paulo, Brazil, Huanglongbing (HLB) is responsible for eradicating 18 million plants between January 2005 and July 2012 (Adami et al., 2014). HLB associated pathogens are part of the Liberibacter genus which is composed of gram-negative, alpha-proteobacteria including the three unculturable, phloem-residing species of *Candidatus* Liberibacter asiaticus, *Candidatus* Liberibacter africanus, and *Candidatus* Liberibacter americanus, which are transmitted by two psyllid vectors: the Asian citrus psyllid (ACP) (*Diaphorina citri* Kuwayama) and the African citrus psyllid (*Trioza erytreae* del Guercio) (J.M. Bové, 2006). *C*Las is propagated by psyllids which preferentially feed on new citrus flushes transmitting the bacteria from infected to non-infected trees or by grafting of infected tissue. In addition to pesticide sprays, current methods to manage the disease involve detection and removal of infected trees while treating orchards with antibiotics, plant hormones, nutritional supplements and thermotherapy (Hoffman et al., 2013; Canales et al., 2016; Munir et al., 2018; Li et al., 2019). These treatments have yielded some effective results, but they are not a cure for the disease; therefore, HLB resistant citrus cultivars are of great interest either

through transgenic citrus or resistant cultivar breeding (Dutt et al., 2015; Ramadugu et al., 2016).

Upon infection of a tree by an insect or grafting, this tree becomes an inoculum for future “clean” psyllids to feed on and spread the bacterium to uninfected trees; therefore, one of the main strategies to limit HLB spread is removal of infected trees. In order to determine if a tree is infected, generally PCR is performed but this method relies on the presence of the bacterial DNA in the sample and due to the uneven distribution as well as the low titer of the pathogen in the tree, detection via this method is inconsistent (Irey et al., 2006; Teixeira et al., 2008; Ding et al., 2016). Furthermore, PCR testing of trees is costly to the growers and government agencies (Li et al., 2006; Arredondo Valdés et al., 2016). Detection of plant responses (including volatile organic compounds, host small RNA’s, and starch accumulation) to infection have shown promise; however these techniques do not directly detect HLB associated pathogens and symptoms of this disease are quite similar to responses caused by other pathogens, nutrient deprivation, or stress (Zhao et al., 2013; Aksenov et al., 2014; Chin et al., 2014; Pourreza et al., 2016).

Due to the necessity of cost efficient and robust detection technologies, our lab screened secreted effectors unique to *C*Las and selected two for serological based recognition of HLB infected plants. CLIBASIA_05315 (SDE1) and CLIBASIA_03230 (SDE2) were predicted from *C*Las strain psy62 genome (GenBank No. CP001677.5). Both proteins are under 20 kDa which would allow

them to travel through the plant companion cells via plasmodesmata, rendering these two proteins good disease markers thus good candidates for antibody production (Balachandran et al., 1997; Imlau et al., 1999). Serological based techniques are commonly used to detect the presence of animal and plant pathogens as these techniques are highly effective and more cost efficient than DNA/RNA based detection assays, which require complex nucleic acid extraction steps as well as expensive laboratory equipment (Slogteren and Slogteren, 1957; Mantur et al., 2007). Direct tissue blot immunoassay (DTBIA) has been developed for citrus tristeza virus (CTV) and *Spiroplasma citri* due to the advantages of serological based assays (Garnsey et al.; Shi et al., 2014). Semi-quantitative RT-PCR using SDE1 and SDE2 specific primers showed that these proteins are expressed in different *C*Las infected citrus species; furthermore, we were able to detect SDE1 transcripts from the asymptomatic citrus seedlings (Pagliaccia et al., 2017). SDE1 and SDE2 also showed higher expression levels (10-fold and 3.5-folds respectively) in infected citrus vs psyllid based on transcriptomic analysis (Yan et al., 2013; Pagliaccia et al., 2017).

In 2012, another member of the Liberibacter genus was sequenced, called *Liberibacter crescens* (Leonard et al., 2012). In 2014, *L. crescens* became the first cultured Liberibacter species (Fagen et al., 2014b). *L. crescens* strain BT-1 (GenBank No. CP003789.1) is a slow-growing, fastidious bacteria with a genome size of 1.5Mb compared to the 1.23 Mb reduced genome of *C*Las (Fagen et al., 2014a). Despite its fastidious nature, *L. crescens* can be cultured on BM7, a

modified BBM medium (Davis et al., 2008; Leonard et al., 2012). *L. crescens* is non-pathogenic but it provides the scientific community with a proxy system (more closely related than previously used Rhizobia) to study the unculturable Liberibacters.

Genomic analysis of *L. crescens* and *C*Las provide insight into the possible supplements required to culture *C*Las; additionally, the ability to grow *L. crescens* may also aid in the effort to culture *C*Las (Leonard et al., 2012; Sena-Vélez et al., 2019). An essential cell surface structure of Gram-negative bacteria is the lipopolysaccharide (LPS) which is critical to maintaining the barrier function preventing the passive diffusion of hydrophobic solutes, such as antibiotics and detergents, into the cell (Zhang et al., 2013). LPS is a large detergent-like molecule comprised of three regions; a highly acylated di-GlcNAc backbone (lipid A) connected to a polysaccharide containing variable repeating sugars (O-antigen) linked through a highly conserved oligosaccharide Kdo/heptose core (Raetz and Whitfield, 2002; Raetz et al., 2007; Wang and Quinn, 2010; Zhang et al., 2013). The O-antigen elicits a strong immunogenic response in mammalian hosts, such as rabbits which are used for antibody production (Frank et al., 1977; Rietschel et al., 1994). In addition to optimizing DTBIA detection using SDE1 antibody, I cultured *L. crescens* for production of two antibodies to improve the detection of HLB infected plants; furthermore, my analysis of LPS results lead to new insight about the differences in structure between pathogenic and non-pathogenic Liberibacters which could aid in culturing of *C*Las.

MATERIALS AND METHODS

Direct tissue blot immunoassay (DTBIA)

Approximately 1-year-old stems were collected from individual citrus trees, for each imprint it was a single tree from throughout the tree. Stem tissues were cut cross-section with a steady motion to obtain a single plane cut surface using a sterile razor blade. The samples were imprinted by gently pressing the freshly cut cross-section of stems on nitrocellulose membranes (Plant Print Diagnostics S.L., Spain), leaving faint green marks from the sap. The printed membranes were dried overnight at 4°C, then washed in TBS-T buffer (0.15M Sodium Chloride, 0.02M Tris-base (pH=7.6) and 0.2% Tween-20) at room temperature for 30 minutes to reduce non-specific binding. Membranes were blocked with 1 x TBS-T containing 5% non-fat milk at 4°C overnight. Primary antibody was prepared by mixing healthy citrus extract (generated by using 1xPBS to grind healthy tissue) with 1:1000 dilution of anti-SDE1 antibody (TBS-T containing 5% fat-free skim milk) overnight. Next day citrus extract and antibody mixture were centrifuged at 1,500 x *g* for 25 minutes at 4°C, then the supernatant was transferred to a new tube and centrifuged 1,500 x *g* for 15 minutes 4°C. Anti-SDE1 antibody was incubated with the membrane for 90 minutes at room temperature shaking at 20 rpm. The membranes were washed two times with TBS-T (10 minutes each time) and washed once with 0.5M EDTA (pH=8.0), for 5 minutes, shaking at room temperature. Membranes were then incubated with the 1:3000 dilution of goat-anti-rabbit IgG-horseradish peroxidase (HRP)-conjugated

secondary antibody (Santa Cruz Biotechnology Inc., United States), for 1 hour at room temperature shaking at 20 rpm. Signals were detected by SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific Inc., United States) following the manufacturer's instructions.

Antibody evaluation of CLas SDE1 using Western blotting

Healthy and infected leaf tissues were ground into fine powder in liquid nitrogen and then suspended in 2x Laemmli buffer (Laemmli, 1970). 2xLaemmli buffer (Laemmli, 1970) was added to all protein extracts and samples were boiled for 5 minutes, then cooled on ice for 5 minutes. Samples were centrifuged at 15,000 x *g* and the supernatant was collected. Supernatants were separated by 12% polyacrylamide gels via SDS-PAGE. Gels were transferred to PVDF membrane paper and blocked with blocking buffer (1xTBS-T (pH=7.4), containing 5% w/v non-fat milk) at room temperature for 1 hour shaking at 20 rpm. Membranes were incubated with anti-SDE1 primary antibody diluted 1:1000 at room temperature for 1 hour shaking at 20 rpm, membranes were washed three times with 1xTBS-T buffer (0.15M Sodium Chloride, 0.02M Tris-base (pH=7.6) and 0.2% Tween-20) for 5 minutes each wash shaking at 20 rpm at room temperature. Membranes were incubated with 1:3000 diluted goat-anti-rabbit IgG-horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotechnology Inc., Unites States) at room temperature for 1 hour shaking at 20 rpm, membranes were washed three times at room temperature with 1xTBS-T

for 10 minutes each wash shaking at 20 rpm. Signals were detected by SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific Inc., United States) following the manufacturer's instructions.

Taq polymerase PCR

PCR and primers were designed as described in (Fagen et al., 2014b). Briefly, primers were designed for specific amplification of the chorismate synthase gene BT-1T (LCF 5'-CGCTCTCGATGGGATTGGAA-3' and LCR 5'-CTGAGGTTTCTG-TCCCCGTC-3'). These primers amplified BT-1 sequences using the following PCR program: 2 minutes at 94ºC; followed by 25 cycles of 94ºC for 20 seconds, 62ºC for 20 seconds, 72ºC for 30 seconds and a final elongation at 72ºC for 5 minutes.

In-house LPS extraction

LPS extraction was performed as described in (Rapicavoli et al., 2018). Briefly, *L. crescens* strain BT-1, and *S. meliloti* strain 1021 were grown on solid media. Cells were collected using 1xPBS (pH=7.4) and centrifuged to concentrate pellet, frozen in liquid nitrogen, then stored at -80ºC. Pellets were washed twice with 1xPBS (pH=7.4) and resuspended in 300µL of resuspension buffer (0.05 M Na_2HPO_4 x $7H_2O$, 0.005M EDTA (pH=7)) with the addition of 40µL Proteinase K (Sigma-Aldrich Inc. United States). Resuspensions were incubated

on rotator at 40 rpm overnight at room temperature. Next day, LPS was extracted using a hot phenol/water method as
described by Marolda and colleagues (Marolda et al., 2006). *E.coli* strain EH100 (Ra mutant) (Sigma-Aldrich Inc. United States) was diluted 1/20 and served as control. Samples were mixed in equal volume with 2x Laemmli buffer (Laemmli, 1970) then boiled for 5 minutes. Samples were centrifuged at 15,000 x *g* and the supernatant was collected. Samples were separated by 18% polyacrylamide gels via SDS-PAGE. After running gels were developed using Silver Stain according to the manufacturer's instructions (Thermo Fisher Scientific Inc., United States).

L. crescens cell antibody generation

L. crescens cells carrying pUFR071::empty vector were grown on solid BM7 media with gentamicin (2µg/mL) for 5-7 days at 28^{0}C (Fleites et al., 2014). 1mg/mL of cells were scraped from the solid BM7 agar plates and resuspended in 1XPBS (pH=7.4). Cells were heat-inactivated by incubating in a 65ºC water bath for 1 hour. After 1 hour, cells were stored at 4ºC and shipped to Pacific Immunology and ProSci for antibody development. Each company injected two New Zealand white rabbits and serum was subsequently IgG purified.

L. crescens LPS glycosyl composition analysis and antibody generation

L. crescens cells carrying pUFR071::empty vector were grown in liquid BM7 media with gentamicin (2µg/mL) for 5-7 days shaking at 150 rpm at 28ºC. Total

amount 1.7L of cells were pelleted by centrifugation in sterile 50 mL tubes at 1,500 x *g* for 10 minutes at 4ºC. Cells were frozen in liquid nitrogen and sent to the CCRC for LPS extraction and glycosyl composition analysis. Mr. Ian Black performed the extraction and composition analysis. LPS extraction was carried out using 90% phenol and water. The sample was mixed with equal volumes buffer (0.05M Na_2HPO_4 x $7H_2O$, 0.005M EDTA, 0.05% NaN_3 (pH=7.0)) and phenol and left to stir at 68°C for 30 minutes. The phenol solution was then chilled on ice, and the sample was transferred to centrifuge bottles and centrifuged at 5,000 x *g* for 20 minutes. The aqueous layer was removed, and buffer (0.05M Na_2HPO_4 x $7H_2O$, 0.005M EDTA, 0.05% NaN_3 (pH=7.0)) was added to the phenol. This process was repeated twice more for a total of three extractions. The aqueous and phenol layers were then dialyzed against distilled water for 3 days in 1-kDa MWCO dialysis bags. The dialyzed samples were freeze dried. The lyophilized fractions were treated with RNase and DNase in 50 mM $MgCl_2$ (pH=7.5) buffer overnight at 37ºC. Proteinase K was added to the sample fractions (50 µg for phenol layer, 400 µg for water layer) and they were again digested overnight. The fully digested sample fractions were then dialyzed again. Extracted material was dissolved in sterile distilled water (~5 mg/mL) and ultracentrifugation was carried out at 100,000 x *g* for 18 hours at 4ºC. A composition analysis was run on the LPS pellet and supernatant to check the purity of the product (Peña et al., 2012; Santander et al., 2013). Sample was freeze dried. I resuspended in filtered sterile distilled water and 1mg/mL was sent

to Pacific Immunology for antibody generation. LPS was conjugated to carrier KLH protein. Two New Zealand white rabbits were injected with the conjugated LPS. Serum was combined from both rabbits and IgG purified.

RESULTS

Antibody evaluation of CLas secreted effectors using DTBIA

To determine the efficiency of anti-SDE1 and anti-SDE2 antibodies in identifying HLB infected trees, fresh cross-section cuts of 1-year-old stems were imprinted on nitrocellulose membranes and then incubated with purified anti-SDE1 antibody generated against SDE1 without secretion signal or purified anti-SDE2 antibodies generated against SDE2 without secretion signal. I printed asymptomatic PCR+ Navel *(C. sinensis)* oranges to compare the efficiency of both antibodies. DTBIA positive signals were strongly detected using anti-SDE1 antibody **(Figure 2.1);** however anti-SDE2 antibody did not specifically detect infected samples instead it non-specifically bound all the tissues **(Figure 2.2).** Having determined anti-SDE1 antibody efficiency in detection of infected trees, we pursued SDE1 as our biomarker. I next evaluated another antibody generated from a peptide of SDE1 called anti-SDE1-peptide-2 (amino acids from Gly^{131} to Met^{150}). While there were signals from asymptomatic HLB infected trees, there also were signals from healthy trees indicating this antibody was not specific **(Figure 2.3)**; therefore, we focused on the antibody generated from the full-length SDE1 protein without secretion signal. Since our focus was to determine a

successful biomarker for detection, I had focused on testing asymptomatic trees; however, I was interested to know if there is a correlation between the intensity of the infection and the ability for anti-SDE1 antibody to detect the difference. I printed samples from asymptomatic and symptomatic trees and incubated them with anti-SDE1 antibody. The results showed weaker signal from the asymptomatic trees than the symptomatic indicating there is a correlation between infection stage and SDE1 production **(Figure 2.4)**.

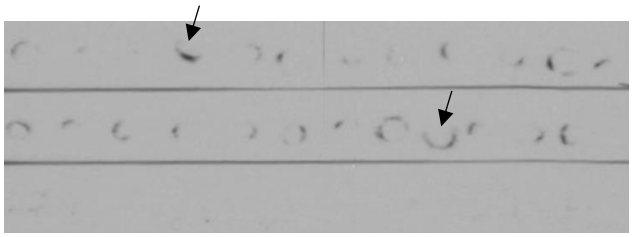

Figure 2.1. Anti-SDE1 antibody evaluation in detection of asymptomatic HLB infected trees. Fresh cross-section cuts of 1-year-old Navel oranges *(C. sinensis)* stems were imprinted on nitrocellulose membranes then incubated with the anti-SDE1 antibody. Positive signals were detected using the direct tissue blot immunoassay.

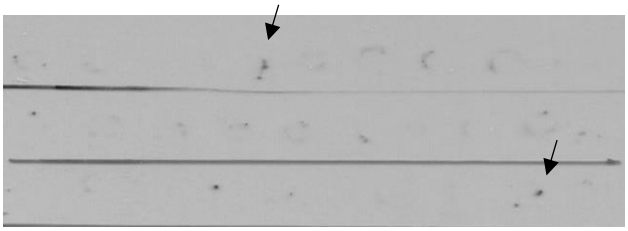

Figure 2.2. Anti-SDE2 evaluation in detection of asymptomatic HLB infected trees. Fresh cross-section cuts of 1-year-old stems from Navel oranges *(C. sinensis)* were imprinted on nitrocellulose membranes then incubated with the anti-SDE2 antibody. Positive signals were detected using the direct tissue blot immunoassay.

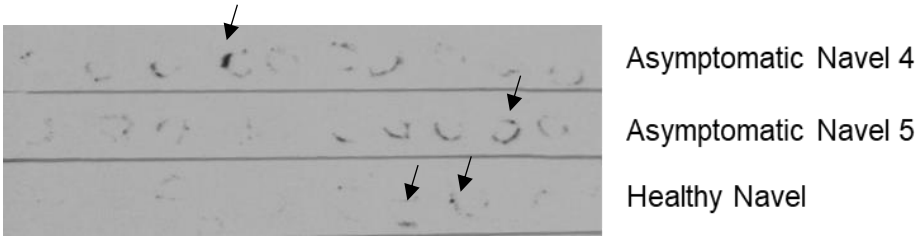

Figure 2.3. Anti-SDE1-peptide-2 evaluation in detection of asymptomatic HLB infected trees. Fresh cross-section cuts of 1-year-old stems from Navel oranges *(C. sinensis)* were imprinted on nitrocellulose membranes then incubated with the anti-SDE1-peptide-2 antibody. Positive signals were detected using the direct tissue blot immunoassay.

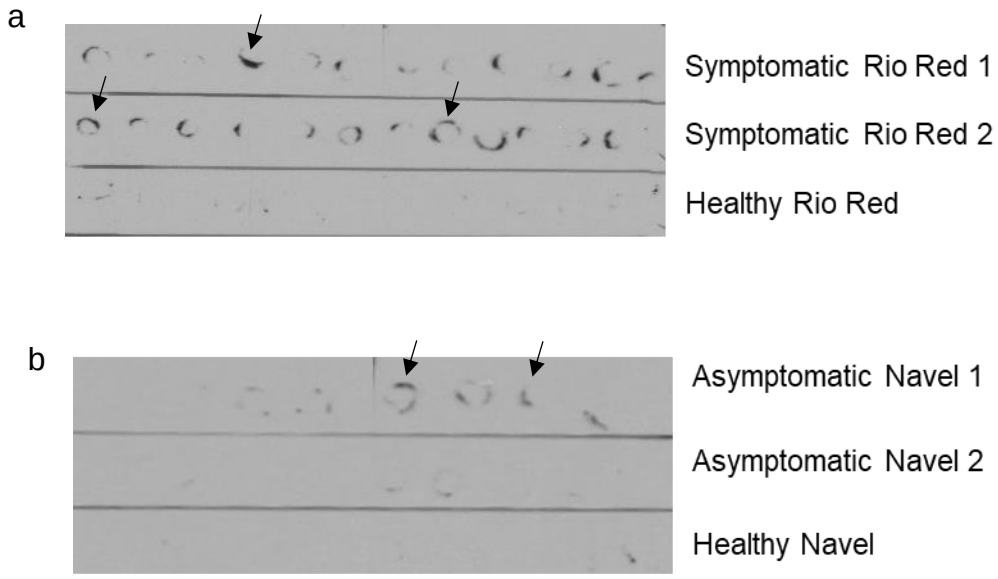

Figure 2.4. Anti-SDE1 antibody evaluation of correlation in detection of (a) symptomatic Rio Red grapefruit (*C. paradisi* Macf.) vs (b) asymptomatic Navel oranges *(C. sinensis)* HLB infected trees. Fresh cross-section cuts of 1-year-old stems were imprinted on nitrocellulose membranes then incubated with the anti-SDE1 antibody. Positive signals were detected using the direct tissue blot immunoassay.

Antibody evaluation of CLas SDE1 using Western blotting

To confirm the efficiency of anti-SDE1 antibody to detect infected tissues, I tested PCR+ trees collected from the Contained Research Facility at UC Davis and a PCR+ tree collected from the Citrus Clonal Protection Program at UC Riverside by Western blotting. SDE1 antibody showed positive signals at the correct predicted size from all the infected samples although some signals from PCR+ samples were weaker than others **(Figure 2.5)**. The healthy control did not show any signal confirming SDE1 binding specificity in Western blotting.

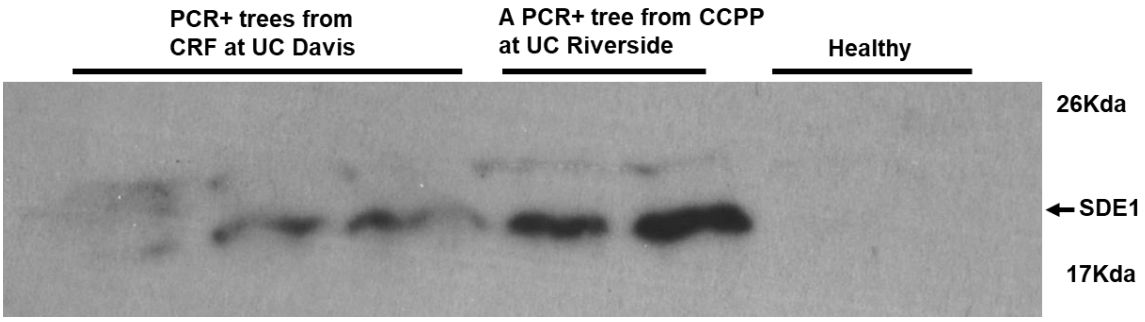

Figure 2.5. Detection of SDE1 from HLB infected plants using Western blotting. Total proteins were extracted from citrus leaves and anti-SDE1 antibody was used to detect positive signals. Arrow indicates the position of SDE1 based on its predicted molecular weight. A similar signal is absent from proteins extracted from healthy tissues.

L. crescens LPS glycosyl composition analysis and antibody generation

When *L. crescens* was cultured in 2014 and multiple labs were able to maintain it in culture, we became involved in a collaborative effort to capture *C*Las cells via microfluidic chambers using cell-surface antibodies. Since these bacteria are close relatives and one of the issues in culturing *C*Las is the initial low titer in citrus hosts, antibodies generated to recognize *L. crescens* cells could capture *C*Las cells and enhance the initial titer before culturing. Furthermore, these antibodies could be used to enhance our detection strategy serving as a cocktail with anti-SDE1. I grew *L. crescens* cells and confirmed BT-1 using primers for chorismate synthase which is unique to *L. crescens* (Fagen et al., 2014b). I included *Agrobacterium tumefaciens* strain GV3101 and water as controls **(Figure 2.6)**. I heat-inactivated *L. crescens* cells and sent them to two companies (Pacific Immunology and ProSci) for antibody production. To further optimize the antibodies, I requested an IgG purification for the sera. The antibodies were tested via ELISA by Dr. Agustina de Francesco and were found to be specific to *L. crescens* but not *Escherichia coli* or *A. tumefaciens*.

Since LPS is on the outermost portion of Gram-negative bacteria and is known to be immunogenic, it was the other antigen we wanted to generate an antibody for. My in-house LPS extract was too contaminated with buffer components for proper analysis and antibody production. Moreover, I used SDS-PAGE developed with Silver Stain using the same protocol and was unable to detect lipid A from *L. crescens* despite successful lipid A extraction from

Sinorhizobia meliloti strain 1021 **(Figure 2.7).** Due to the need of specialized equipment to carry out the extraction, we purchased an LPS extraction and glycosyl composition analysis from the CCRC. The lyophilized LPS was sent back to us and I sent it for antibody production at Pacific Immunology. The sera were also IgG purified for this antibody. Both antibodies are currently being used for robust detection of HLB via ELISA in our lab, visualization of *C*Las movement in planta by Dr. Amit Levy at University of Florida, and to confirm *C*Las growth in an *in vitro* culture by Professor Dean W. Gabriel at University of Florida. The glycosyl composition analysis showed that *L. crescens* LPS contained mostly ribose, smaller amounts of rhamnose, galactose and glucose **(Figure 2.8).** LPS also contained beta hydroxyl fatty acids: C14:OH, C16:OH and C18:OH, which are present in the anchor part of lipid A **(Figure 2.9)**. 2-Keto-3-deoxy-octonate (KDO) and heptose were also detected. Interestingly the presence of 27OHC28:0, a very long chain fatty acid (VLCFA), was determined to be part of *L. crescens* LPS **(Figure 2.10)**.

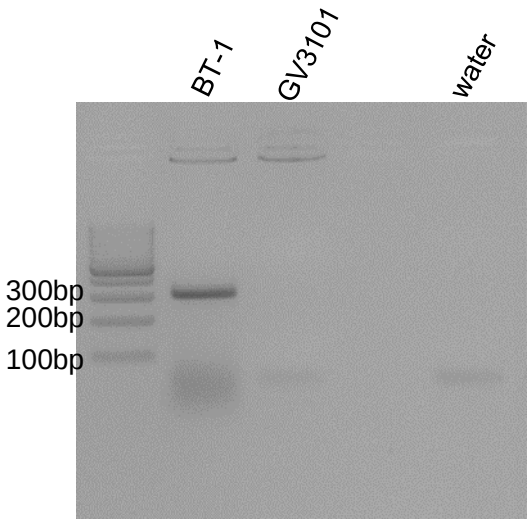

Figure 2.6. Chorismate synthase product only in *Liberibacter crescens* BT-1 but not controls. *Taq* polymerase PCR product using chorismate synthase primers with predicted product size at 288bp. *L. crescens* strain BT-1 shows product at the correct size while *A. tumefaciens* stain GV3101 does not ensuring the culture is BT-1. Master mix excluding DNA template serves as water control.

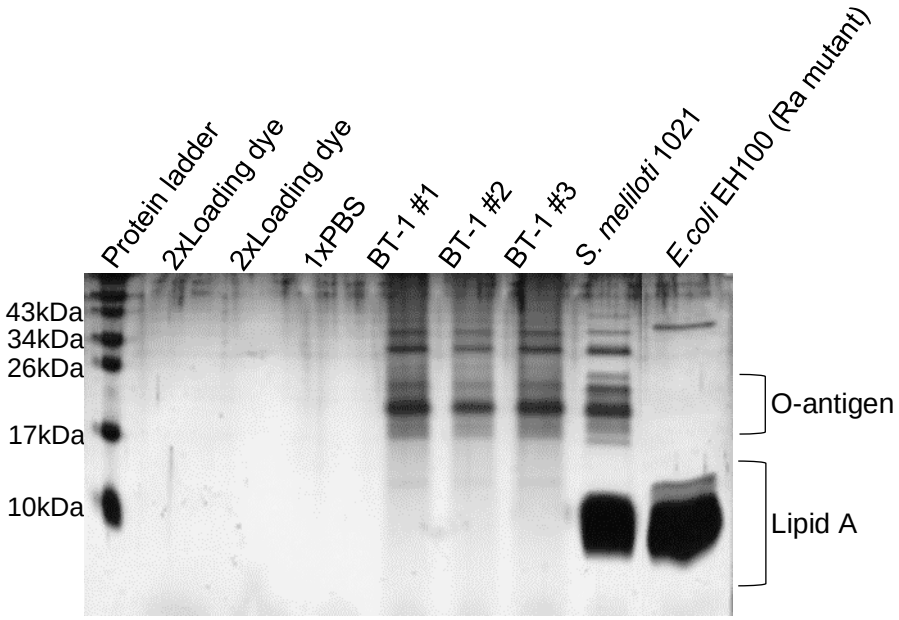

Figure 2.7. In-house LPS extraction did not successfully extract lipid A of *Liberibacter crescens*. LPS purified from *L. crescens* strain BT-1 from different cultures as well as *S. meliloti* strain 1021. Sigma-Aldrich purified *E.coli* strain EH100 (Ra mutant) produces a rough LPS without O-antigen. Samples were run on 18% SDS-PAGE and Silver Stained.

Sample	Glycosyl residue	Mass (µg)	Mol %
LPS pellet	Ribose (Rib)	65.7	58.4
	Arabinose (Ara)	n.d.	-
	Rhamnose (Rha)	0.7	0.6
	Fucose (Fuc)	n.d.	-
	Xylose (Xyl)	n.d.	-
	Glucuronic Acid (GlcA)	n.d.	-
	Galacturonic acid (GalA)	n.d.	-
	Mannose (Man)	n.d.	-
	Galactose (Gal)	43.3	32.0
	Glucose (Glc)	9.0	6.7
	N-Acetyl Galactosamine (GalNAc)	n.d.	-
	N-Acetyl Glucosamine (GlcNAc)	3.9	2.3

Figure 2.8. The glycosyl composition analysis showed that *Liberibacter crescens* LPS contained mostly ribose, smaller amounts of rhamnose, galactose and glucose. Summary of GC/MS chromatogram of the TMS methyl glycosides produced from the ultracentrifuge supernatant fraction. The estimated weight and mole percentage of each detected monosaccharides in the LPS.

Fatty Acid	Area %
C14:OH	18.3
C16:OH	53.8
C18:OH	3.4
27OHC28:0	24.5

Figure 2.9. LPS of *Liberibacter crescens* contained beta hydroxyl fatty acids: C14:OH, C16:OH and C18:OH. Summary of GC/MS chromatogram of the TMS methyl glycosides produced from the ultracentrifuge supernatant fraction.

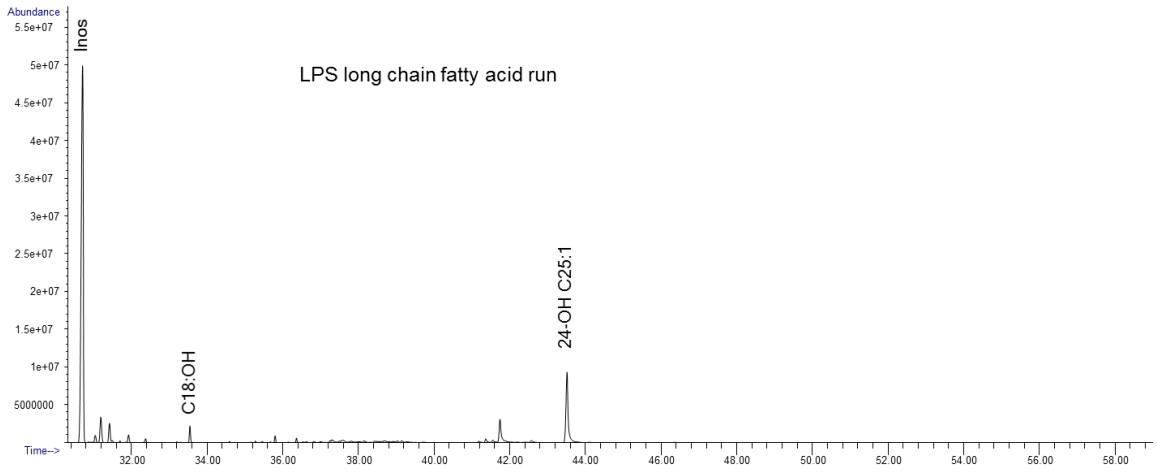

Figure 2.10. LPS of *Liberibacter crescens* contains a late eluting peak that corresponds to a C28 fatty acid and hydroxyl group at carbon 27 (27OHC28:0). Long GC run of the LPS sample that allows for the visualization of longer chain fatty acids.

DISCUSSION

In the United States of America, Huanglongbing has been detected in three significant citrus producing states (da Graça et al., 2016): Florida (Halbert, 2005), Texas (Kunta et al., 2012; da Graça et al., 2015) and California (Kumagai et al., 2013) as well as in South Carolina, Georgia and Louisiana (Halbert et al., 2010). The recommended measures for HLB management are based on two phytopathologically sound principles: inoculum reduction by frequent removal of HLB affected trees and control of psyllid vector populations by insecticide treatment (Belasque Jr et al., 2012). Therefore, identification of infected HLB trees is a key component of any HLB management. Evaluating *C*Las biomarkers for detection using direct tissue blot imprints immunoassay (DTBIA) showed that in fact some effectors may be useful in this effort. Based on my own experiences on this project, seasonal, environmental, and horticultural conditions including fertilization and irrigation produce succulent stems ideal for imprinting. When conditions are unfavorable as is commonly found in the field, stems are difficult to imprint; therefore, DTBIA efficiency is influenced by conditions other than lab protocol. Conveniently my effort in antibody development and confirmation aided in another detection platform called enzyme-linked immunosorbent assay (ELISA) (Pagliaccia et al., 2017). In the ELISA method, plant extracts are bound to a specialized 96-well plate and then developed using primary and secondary antibodies (Hnasko, 2015). In this case, less succulent plants that were challenging to imprint now can be tested for HLB infection using ELISA while

employing anti-SDE1 antibody which was proven to be a useful antibody. Anti-SDE1 antibody was also successful at detecting infected tissue using Western blotting. Using anti-SDE1 antibody my previous lab member, Dr. Deborah Pagliaccia, detected a weaker signal from asymptomatic than symptomatic tissues in Western blotting (Pagliaccia et al., 2017). Her results correlated with my DTBIA data showing weaker signals from imprints of asymptomatic trees. These results suggested: (1) SDE1 proteins accumulate during disease progression and/or with increasing bacterial titer; (2) SDE1 proteins may be present in citrus tissues independent of *C*Las cells; and (3) SDE1 is likely produced at an early infection stage (Pagliaccia et al., 2017) .

Our initial focus to study *L. crescens* was for the generation of cell-surface antibodies to capture *C*Las cells in microfluidic chambers and increase bacterial titer in sample preparation. This collaboration led us to generate antibodies that can be used along with anti-SDE1 antibody creating a cocktail. Moreover, LPS structural analysis resulted in the discovery of a very interesting difference between *L. crescens* and *C*Las. *L. crescens* contains a very long chain fatty acid (VLCFA 27OHC28:0). LPS from *L. crescens* is similar to relatives in the Rhizobium genus. Long acyl chains are present in the lipid A molecules of all members of the Rhizobiaceae family (Bhat et al., 1991; Basu et al., 2002). *E. coli* and Rhizobia utilize the same biochemical pathway to synthesize the lipid A precursor molecule Kdo2-lipid-IVA from uridine diphosphate N-acetylglucosamine (UDP-GlcNAc). In later steps of LPS synthesis, the *E. coli* and

Rhizobia pathways diverge, and Rhizobia produce a lipid A that is structurally very different from that of *E. coli* (Raetz et al., 2007). Among the differences in the lipid A structures is the nature of the secondary fatty acids, such as the acyloxyacyl residues. *E. coli* adds two acyloxyacyl fatty acids, namely lauryl and myristyl residues, to the Kdo2-lipid-IVA; whereas, Rhizobia put on a single, extra-long fatty acid residue, 27- hydroxyoctacosanoic acid (27OHC28:0) (Vedam et al., 2004). To accomplish this, Rhizobia require a specialized acyl carrier protein, AcpXL and a minimum of one specific transferase, LpxXL (Vedam et al., 2003). LpxXL is distantly related to the lauroyltransferase (LpxL) of *E. coli* required for lipid A biosynthesis, but highly significant LpxXL orthologues are present in *A. tumefaciens*, *Brucella melitensis*, and all sequenced strains of Rhizobia, consistent with the occurrence of long secondary acyl chains in the lipid A of these organisms (Basu et al., 2002). Interestingly, our collaborator Professor Dean W. Gabriel attempted to generate a *C*Las-like *L. crescens* by knocking out LpxL which is present in the LPS pathway of *L. crescens* and *S. meliloti* but missing from *C*Las, *C*Lam, and *C*Laf. Their attempts had failed without a technical reason. Intriguingly, research suggests this VLCFA component may play a role in hiding from immunity unlike Gram-negative pathogens that do not produce it (Que, 2000; Que et al., 2000; Basu et al., 2002).

My work in this chapter highlights the use of *C*Las biomarker SDE1 as well as *L. crescens* cell surface components for antibody generation and HLB detection. Additionally, *L. crescens* cell and LPS antibodies are being used for *in planta*

movement visualization of *C*Las by Dr. Amit Levy at University of Florida, and for confirmation of *C*Las maintenance and growth *in vitro* by Professor Dean W. Gabriel at University of Florida. Finally, our investigation into the structure of LPS led to a focal piece of data allowing me to synthesize a hypothesis clarifying results from multiple labs regarding the LPS pathway in *C*Las and *L. crescens*. This VLCFA component may be involved in bacterial culturing as well as plant-immunity deception.

ACKNOWLEDGEMENTS

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Chapter 3

Development of *Liberibacter crescens* as a tool to study Sec-delivered effectors of *Candidatus* Liberibacter asiaticus

ABSTRACT

Huanglongbing is currently the most destructive citrus disease worldwide and it is associated with three unculturable species *Candidatus* Liberibacter asiaticus (*C*Las), *Candidatus* Liberibacter africanus (*C*Laf), *Candidatus* Liberibacter americanus (*C*Lam). *Liberibacter crescens* (*L. crescens*) is the closest culturable relative of the HLB associated bacteria and it can be genetically modified. *L. crescens* can be used to study certain aspects of *C*Las pathogenesis. Bacterial pathogens often employ secreted protein effectors to facilitate infection. *C*Las has been found to encode predicted secreted effectors, but these cannot be confirmed via experimentation due to lack of culturability of *C*Las. Currently, the prevailing mechanism to identify possible secreted effectors of *C*Las rely upon bioinformatics software designed to detect the N-terminal secretory signal. In this chapter, I generated a functional model system to confirm predicted *C*Las secreted effectors using *L. crescens*. Our lab generated a list of predicted secreted effectors of *C*Las and we focused on some to serve as HLB detection biomarkers. Functional work was performed on one of these effectors SDE1 (CLIBASIA_05315). Using this foundation of knowledge, I used SDE1 as my test subject for secretion in *L. crescens*. My research shows that *L. crescens* can serve as a tool to confirm and possibly study *C*Las secreted effectors.

INTRODUCTION

Upon genome sequencing it was discovered that both *Candidatus* Liberibacter asiaticus (*C*Las) and *Liberibacter crescens* (*L. crescens)* contain genes for the general Sec secretion system (Duan et al., 2009; Fagen et al., 2014). In Gram-negative bacteria, proteins destined for Sec secretion are recognized and bound by chaperone SecB at their removable N-terminal secretory signal sequence, preventing the pre-secretory protein from folding (Ullers et al., 2004; Randall and Hardy, 2002; Green and Mecsas, 2016). SecB then delivers its substrates to SecA, a multi-functional protein that both guides proteins to the protein-conducting channel SecYEG, and also serves as the ATPase that provides the energy for protein translocation (Manting et al., 2000; Scheuring et al., 2005; Mitra et al., 2005; Breyton et al., 2002; Hartl et al., 1990). Before transporting the protein, a peptidase cleaves off the SecB signal sequence from the protein, and the secreted protein is then folded upon delivery to the periplasm (Mogensen and Otzen, 2005). While many proteins delivered by the SecB system remain in the periplasm, some will ultimately become extracellular (Green and Mecsas, 2016). Transmembrane proteins are also secreted through SecYEG channel. Proteins destined to embed in the bacterial membranes reasonably contain hydrophobic domains, so they are unstable in the cytoplasm. The signal recognition particle (SRP) binds to the pre-transmembrane proteins and delivers its substrates to FtsY, which translocates the ribosome-protein complex to the SecYEG channel (Schierle et al., 2003;

Sijbrandi et al., 2003; Luirink and Sinning, 2004).

Secreted protein effectors (here on effectors) of bacteria have been well-studied to elucidate the use of bacterial made proteins that are delivered to the host in order to facilitate infection. HLB associated bacteria are intracellular pathogens that are transferred to plant cells via the stylet structure of psyllid during feeding; therefore, it is reasonable that these pathogenic Liberibacters do not encode a complex injection apparatus commonly used to deliver effectors into host cells (Duan et al., 2009). Moreover, effectors of similar intracellular, insect-vectored Phytoplasmas have been shown to secrete effectors using the Sec secretion system (Bai et al., 2009; Hoshi et al., 2009; Sugio et al., 2011). Phytoplasmas lack a cell wall, so their membrane proteins and secreted proteins function directly in the cytoplasm of the host plant or insect cell (Hogenhout et al., 2008). *C*Las, however contains an outer membrane that Sec secreted effectors must bypass before reaching the host. Due to the possible function of effectors in virulence and the Gram-negative structure of *C*Las, it is of interest to analyze what proteins in HLB associated bacteria are secreted and how they leave the cell.

Currently, the method of screening for *C*Las secreted effector relies on bioinformatics software designed to detect the protein's N-terminal secretory signal since it has conserved features. A typical signal peptide is between 20 and 30 amino acids and has a three-domain structure: an N-terminal domain with 1-8 positively charged residues, followed by a helical hydrophobic core of 4-16

residues (linked amino acids) and a slightly polar C-terminal domain containing the signal peptidase cleavage site (von Heijne, 1985; Gierasch, 1989; Chatzi et al., 2013). Proteins predicted to be secreted from *C*Las have been screened *in vivo* by fusing their N-terminal secretory signals to a reporter gene in *Escherichia coli (E. coli)* (Prasad et al., 2016). When *L. crescens* was cultured, scientists focused on transforming it in the hopes of generating a proxy system to study *C*Las. They succeeded and stably transformed naturally competent *L. crescens* using pUFR071, a wide bacterial host range (repW) replicon (Fleites et al., 2014; Jain et al., 2019). Using *L. crescens,* it was confirmed that prophage peroxidases predicted to be providing *C*Las defense against ROS production do in fact provide *L. crescens* with the ability to tolerate H_2O_2 better than controls (Jain et al., 2015). Naranjo and colleagues utilized *L. crescens* for another perspective, they studied previously made observation of *Candidatus* Liberibacter spp.' (*C*Ls) biofilm formation (Cicero et al., 2017). They discovered that *L. crescens* attaches to surfaces, and form cell aggregates embedded in a polysaccharide matrix in microfluidic chambers (Naranjo et al., 2019). Biofilm structures may represent excellent adaptive advantages for *C*Las during insect vector colonization helping with host retention, immune system evasion, and transmission (Naranjo et al., 2019). These examples highlight the capability of *L. crescens* as a functional tool to study *C*Las. Considering the fastidious nature of the HLB associated pathogens, it is likely even after the bacteria are cultured it will take significant time and effort to develop it into a genetically modifiable system to study bacterial

pathogenesis.

In order to generate a reliable system to directly confirm predicted effectors are being secreted by *C*Las, I generated a functional model system in *L. crescens*. I screened for a functional promoter by searching for constitutively expressed genes in the *L. crescens* genome. Using the nucleotide sequences of genes predicted to be constitutively expressed based on function, I generated a codon usage table for sequence optimization of *L. crescens* expression (Countcodon program: https://www.kazusa.or.jp/codon/countcodon.html). I designed multiple promoters and Shine-Dalgarno sequences upstream of codon optimized green fluorescent protein (GFP) as a reporter for a functional promoter. I screened four promoters and identified two to be functional as well as two Shine-Dalgarno (Shine and Dalgarno, 1974) sequences from *L. crescens* genome and proceeded to synthesize a construct with promoter 1 upstream of SDE1 (Sec-Delivered Effector 1) (Twist Bioscience). I confirmed that *L. crescens* can express and secrete SDE1 upon mixture with citrus extract. Furthermore, I tested *C*Las protein CLIBASIA_00520 which is predicted to be secreted (based on its N-terminal sequence) while also predicted to function as a lipoprotein remaining hinged to the outer membrane by the software PROSITE (Hulo, 2006). CLIBASIA_00520 was not secreted upon incubation of *L. crescens* with citrus extract indicating *L. crescens* can serve as a useful system to confirm predicted characteristics of *C*Las genes.

MATERIALS AND METHODS

Functional promoter generation

L. crescens genome was analyzed for bacterial genes with housekeeping function. Upstream regions of varying lengths were selected including two Shine-Dalgarno (SD) sequences to function as ribosome binding sequences (RBS), AGGAA and AGGAG. Promoter #1 is 168 base pairs upstream of a DNA-directed RNA polymerase beta subunit (B488_08440), encoded on the reverse strand from 925123-929268 with AGGAG as the predicted ribosome binding site. Promoter #2 was 76 base pairs upstream of a small subunit ribosomal protein S4 (B488_05260) encoded on the forward strand from 568629-569246 with AGGAA as the predicted ribosome binding site. Promoter #3 is 156 base pairs upstream of the same small subunit ribosomal protein S4 (B488_05260) as promoter #2. Promoter #4 was promoter #3 linked to the Sec secretion signal from SDE1. The codon usage table was generated using 70 genes predicted to be constitutively expressed based on functional homology with other bacteria. Gene sequences were concatenated and input into a Countcodon software version 4 (https://www.kazusa.or.jp/codon/countcodon.html). The resulting table was used by Twist Bioscience to optimize GFP, SDE1 and CLIBASIA_00520-6xHIS sequences. Synthesized fragments were cloned (https://www.addgene.org/protocols/subcloning) into pUFR071 (Fleites et al., 2014) then transformed into *E. coli* DH5α (New England Biolabs Inc., United States) for verification and plasmid extraction.

Protein extraction and Western blotting

L. crescens cultures were grown 5-7 days in liquid BM7 medium containing gentamicin (2µg/mL) at 28°C shaking at 150 rpm. Cells were collected at 15,000 x *g* for 5 minutes at 4ºC. 2xLaemmli buffer (Laemmli, 1970) was added to all protein extracts and samples were boiled for 5 minutes, then cooled on ice for 5 minutes. Samples were centrifuged at 15,000 x *g* and the supernatant was collected. Supernatants were separated by 12% or 15% polyacrylamide gels via SDS-PAGE. Gels were transferred to PVDF membrane paper and blocked with blocking buffer (1xTBS-T (pH=7.4), containing 5% w/v non-fat milk) at room temperature for 1 hour shaking at 20 rpm. Membranes were incubated with primary antibody at room temperature for 1 hour shaking at 20 rpm, membranes were washed three times with 1xTBS-T buffer (0.15M Sodium Chloride, 0.02M Tris-base (pH=7.6) and 0.2% Tween-20) at room temperature for 5 minutes each wash shaking at 20 rpm. Membranes were incubated with secondary antibody at room temperature for 1 hour shaking at 20 rpm. Membranes were washed three times at room temperature with 1xTBS-T for 10 minutes each wash shaking at 20 rpm. Signals were detected by SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific Inc., United States) following the manufacturer's instructions. Below are the details for the three proteins I detected using Western blotting, the described protocol applies to all.

1) For GFP functional promoter screening, total proteins were separated by 12% SDS-PAGE, incubated with primary antibody anti-GFP at 1:2000 dilution (Clontech Laboratories Inc., United States) and 1:4000 dilution secondary antibody goat-anti-mouse IgG-horseradish peroxidase (HRP)-conjugated (Santa Cruz Biotechnology Inc., United States).

2) For SDE1 expression total proteins were separated by 15% SDS–PAGE, incubated with primary antibody anti-SDE1 at 1:1000 dilution, and secondary goat-anti-rabbit IgG-horseradish peroxidase (HRP)-conjugated secondary antibody 1:4000 dilution (Santa Cruz Biotechnology Inc., United States).

3) For CLIBASIA_00520-6xHIS expression total proteins were separated by 15% SDS-PAGE, incubated with 1:2000 dilution of His-Horseradish Peroxidase (HRP)-conjugated Antibody (R&D Systems Inc., United States).

Liberibacter crescens electroporation

As described in Fleites et al. (2014), *L. crescens* cultures were grown 5-7 days in liquid BM7 medium at 28°C shaking at 150 rpm until OD_{600} reached 0.65. Cells were chilled on ice for 30 minutes and collected by centrifugation at

1,500 x *g* for 15 minutes at 4ºC. Bacterial pellets were rinsed twice in 20 mL of ice-cold sterile water then resuspended in 2 mL of ice-cold 10% glycerol then flash-frozen in liquid nitrogen and stored at -80ºC. 40 µL of *L. crescens* competent cells (Fleites et al., 2014) were electroporated at 1800V (Eppendorf Eporator System, 100-240 VAC, 50/60Hz) with approximately 500ng of plasmid DNA. The cells were recovered in 900 µL of BM7 medium without antibiotics and allowed to recuperate for 16 hours with gentle shaking (150rpm at 29ºC). After recovery, *L. crescens* cells were plated on BM7 agar media containing gentamicin (2µg/mL).

Osmotic Shock

Osmotic shock was performed according to (Life Technologies Inc., United States). *L. crescens* cells were under a constitutive promoter so no induction of expression was required. *L. crescens* cultures were grown 5-7 days in liquid BM7 medium containing gentamicin (2µg/mL) at 28°C shaking at 150 rpm. Bacterial cells were harvested by centrifugation at 15000g and resuspended in Osmotic Shock Solution 1 (20mM Tris-HCl, 2.5mM EDTA, 20% sucrose at pH=8). Cells were incubated on ice for 10 minutes then collected by centrifugation at 15000g for 1 minute at 4ºC and the supernatant was removed. Pellets were resuspended in Osmotic Shock Solution 2 (20mM Tris-HCl, 2.5mM EDTA) and incubated for 10 minutes on ice. Afterwards, the cells were centrifuged for 10 minutes 15000g at 4ºC. Supernatant (shock fluid) was collected and kept on ice. Pellets were

resuspended in Osmotic Shock Solution 2. Supernatant (shock fluid) was analyzed along with cell pellets to determine if secreted proteins were present in the periplasm. Pellet and supernatants were evaluated SDS-PAGE and Western blotting.

Induction using Citrus Extract

Protocol modified by Dr. Deborah Pagliaccia and Tyler Dang from Hijaz and Killiny, 2014. Green bark from Oroblanco grapefruit (*Citrus grandis* Osbeck x *C. Paradisi* Macf.) was surface sterilized, peeled, sliced on the abaxial side vertically and chopped up. The chopped citrus was loaded into 25mL Nonsterile Centrifugal Filters (0.45µM cellulose acetate filters) (Thermo Fisher Inc., United States). Tubes were flash frozen in liquid nitrogen then spun down at 10,000 x *g* for 1 hour at 4ºC. Meanwhile, 3 mL 1-week-old *L. crescens* cultures were collected at 15,000 x *g* for 5 minutes at 4ºC and cells were resuspended in 500µL of 0.3M sucrose supplemented with 250µL of citrus extract, after they were mixed well, samples were incubated at 28ºC without shaking for 24 hours (Shi et al., 2014). The following day, cells were collected at 15,000 x *g* for 5 minutes at 4ºC. Supernatant were precipitated using the Pyrogallol red-molybdate-methanol PRMM protocol (Caldwell and Lattemann, 2004). Cell pellet was resuspended in 150µL 2xLaemmli (Laemmli, 1970) buffer then boiled for 5 minutes then cooled on ice for 5 minutes. Proteins were separated by SDS-PAGE and visualized by Western blotting.

Pyrogallol red-molybdate-methanol (PRMM) Protein Precipitation

Considering the slow-growing (fastidious) nature of *L. crescens* I predicted there may not be a high enough concentration of proteins secreted to be detected in the supernatant. Therefore**,** supernatant samples were precipitated using pyrogallol red-molybdate-methanol (PRMM) precipitation method (Caldwell and Lattemann, 2004). Protein precipitation was performed using pyrogallol red-molybdate-methanol (PRMM) as described in Caldwell and Lattemann 2003. Equal volumes of the *L. crescens* cell supernatant and PRMM solution were mixed (0.05 mM pyrogallol red, 0.16 mM sodium molybdate, 1.0 mM sodium oxalate, 50.0 mM succinic acid, 20% methanol in H_20 adjusted to pH=2.0 with HCl) and incubated at room temperature for one hour, followed by overnight incubation at 4ºC. Next day, samples were centrifuged at 15,000 x *g* for 30 minutes at 4ºC. Supernatants were discarded and the pellet was rinsed with ice-cold acetone. Samples were centrifuged a second time at 15,000 x *g* for 10 minutes at 4ºC. Samples were air dried for 10 minutes and resuspended in 150µL 2xLaemmli (Laemmli, 1970) buffer then boiled for 5 minutes then cooled on ice for 5 minutes. Proteins were separated by SDS-PAGE and visualized by Western blotting.

RESULTS

Functional L. crescens promoter screening

To develop a functional system for protein expression and secretion in *L. crescens*, I searched for essential components to complete our vector pUFR071. I selected multiple possible promoters from upstream regions of constitutively expressed genes in the *L. crescens* strain BT-1 genome (GenBank No. CP003789.1). I synthesized GFP reporter protein downstream of the four promoters to screen for a functional option **(Table 3.1)**. A codon usage table was generated for *L. crescens* **(Figure 3.1)** and utilized by Twist Bioscience to synthesize the promoters upstream of GFP **(Figure 3.2a)**. Using Western blotting I observed GFP protein expression using Promoter #1 and Promoter #2 only. Promoter #1 showed stronger GFP expression than promoter #2, so we decided to pursue promoter #1 for *L. crescens* protein expression and secretion study **(Figure 3.2c).**

[Triplet] [frequency: per thousand] ([number])

UUU 31.3(765) UCU 32.5(795) UAU 23.1(565) UGU 8.5(207)

UUC 6.1(148) UCC 5.1(125) UAC 3.5(85) UGC 1.6(38)

UUA 25.6(625) UCA 16.5(403) UAA 2.0(50) UGA 0.4(10)

UUG 15.7(384) UCG 3.3(81) UAG 0.4(10) UGG 6.0(146)

CUU 30.1(736) CCU 18.8(459) CAU 17.4(425) CGU 31.3(764)

CUC 5.2(127) CCC 2.0(50) CAC 2.7(66) CGC 6.1(150)

CUA 5.4(132) CCA 12.6(309) CAA 24.4(596) CGA 7.1(174)

CUG 4.3(104) CCG 3.8(93) CAG 12.5(306) CGG 3.2(77)

AUU 46.7(1141) ACU 17.0(416) AAU 39.2(958) AGU 10.0(245)

AUC 10.0(245) ACC 4.3(104) AAC 8.1(198) AGC 4.6(112)

AUA 26.9(657) ACA 19.9(486) AAA 47.3(1155) AGA 9.2(225)

AUG 23.9(585) ACG 4.8(117) AAG 22.9(560) AGG 3.8(94)

GUU 39.1(956) GCU 32.7(800) GAU 49.4(1208) GGU 33.1(808)

GUC 6.2(152) GCC 6.6(162) GAC 7.2(176) GGC 7.9(194)

GUA 17.5(427) GCA 22.3(545) GAA 51.2(1251) GGA 22.8(558)

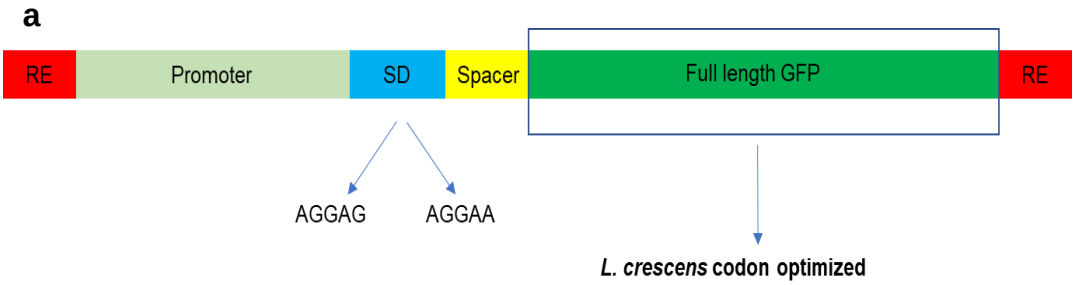
GUG 6.5(160) GCG 4.8(118) GAG 18.7(458) GGG 6.7(164)

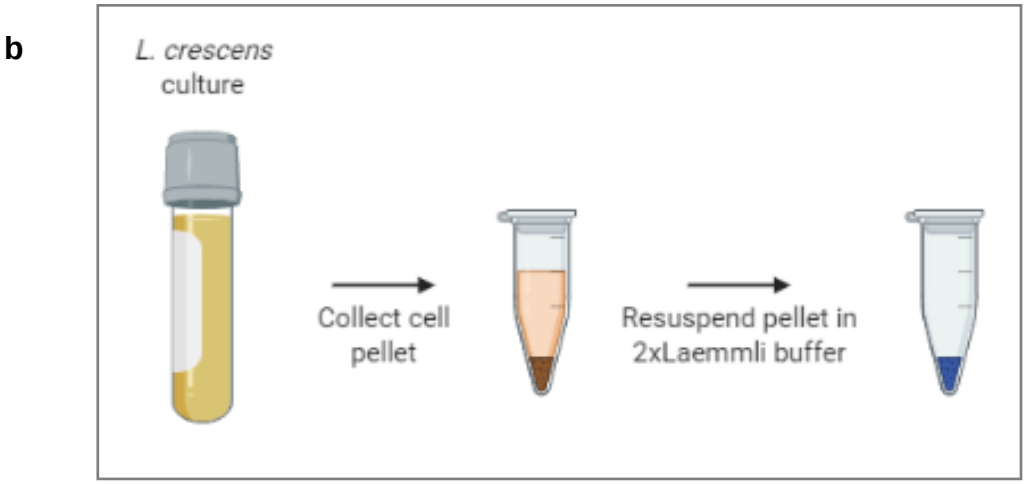
Figure 3.1. *Liberibacter crescens* codon usage. Nucleotide sequences from 70 *L. crescens* genes were concatenated and input into (Countcodon program version: https://www.kazusa.or.jp/codon/countcodon.html).

Table 3.1. Codon optimized nucleotide sequences for promoters, GFP, SDE1 and CLIBASIA_00520 sequences.

Name	Sequence
Promoter #1	TAAATATATAAGCTAATGTTTGGTTATTGTTTGTTTAGGTAGAGATGTATTTTGTTTTTGTTTTT TAACTGTTTCAAAAGCATATTGATTGATTATCTCTTTCATAAAAATATTAACTGAAGCTTTCTATT TAATTTAAGCATGATAAAAAGTTAGTA**AGGAG***CGACG*
Promoter #2	TCATGTCAAAGGAGGGCGCGTTTCCTTGAGATACGTCTTGTTAATGTTATGAAATCATTAAATTA A**AGGAA***ATGCG*
Promoter #3	TTTTGGTATGTTTCCTGTTTATTAAACGTGTCCCGTGGTCATTCCTGTCAAAAGGTTAGTGGCC TGTCAGAAATGAGAGATCATGTCAAAGGAGGGCGCGTTTCCTTGAGATACGTCTTGTTAATGTT ATGAAATCATTAAATTAA**AGGAA***ATGCG*
Promoter #4-Sec-signal	TTTTGGTATGTTTCCTGTTTATTAAACGTGTCCCGTGGTCATTCCTGTCAAAAGGTTAGTGGCC TGTCAGAAATGAGAGATCATGTCAAAGGAGGGCGCGTTTCCTTGAGATACGTCTTGTTAATGTT ATGAAATCATTAAATTAA**AGGAA***ATGCG* ATGAAGCGTTTTCTTATGTTATCTCTTCTTGCTTCTA CAGCAAATTTTGCTTATGCA
GFP	ATGGTATCAAAAGGTGAAGAATTATTTACTGGTGTTGTTCCAATTCTTGTTGAATTAGATGGTGA TGTTAATGGACATAAATTTTCTGTATCAGGTGAGGGTGAAGGTGATGCAACTTATGGTAAATTAA CTTTGAAGTTTATATGTACGACAGGCAAATTGCCTGTTCCATGGCCTACATTAGTTACAACGCTT ACGTATGGTGTACAATGTTTCTCTCGTTATCCTGATCATATGAAACAACATGATTTTTTCAAATC TGCTATGCCAGAAGGTTATGTACAGGAACGTACGATTTTTTTTAAGGATGATGGTAATTATAAAA CACGTGCAGAGGTTAAATTTGAAGGAGATACATTAGTTAATCGTATAGAGCTTAAAGGTATCGAT TTTAAGGAGGATGGTAATATTTTAGGCCATAAATTAGAATACAACTATAACTCTCACAATGTTTAT ATAATGGCAGATAAACAGAAGAATGGAATTAAAGTAAACTTTAAGATACGTCACAATATAGAAGAT GGAAGTGTTCAGCTTGCTGATCATTATCAACAGAATACACCTATCGGAGATGGTCCAGTTTTGT TACCTGATAATCATTACCTTTCAACACAGAGTGCACTTTCAAAAGATCCAAATGAAAAACGTGACC ATATGGTTCTTTTGGAATTCGTTACTGCTGCTGGCATTACGTTGGGTATGGATGAGTTGTATAA GTAG
Full-length SDE1	GTTCGTAAAAATTTGTTAACATCAACTTCTTCTCTTATGTTTTTTTTTCTTTCTTCAGGTTATGC TTTGTCTGGTTCTTCTTTTGGTTGTTGTGGTGAGTTTAAAAAAAAAGCTTCTTCTCCTCGAATT CATATGCGCCCTTTTACTAAATCTTCACCATATAATAATAGTGTTTCTAATACTGTAAATAATACT CCTCGCGTTCCGGATGTATCTGAAATGAATTCTTCACGTGGTTCAGCACCTCAGTCTCATGTTA ATGTTTCTTCACCACATTATAAACACGAGTATTCTTCAAGTTCAGCATCTTCTAGTACACACGCA AGTCCGCCTCCTCATTTTGAACAGAAACATATTTCTCGAACTCGTATTGATTCTTCTCCTCCTCC AGGACATATTGATCCTCACCCGGATCATATTCGTAATACTTTAGCTCTTCATAGAAAGATGCTTG AACAATCTTAA
Full-length CLIBASIA_00520-6xHIS	ATGCATTTTAAGATTAAACGCTTTCTTTTCCCACTTTTGGCTCTTCTTGGCAGTTGTGATGATAA TCCAAAAGATCCAATTGTTCAATTTAAACAAATGAAATATGAATCTCAAGAATCAAAAAAATCATTA TCTGATGCTTTATTTAAAACTTACCCAGATACAATGGATAAGATTAATACAGTTCAGACAGCTCTT AGAAACTTACATAATGCTATAAGTAAAATGGAATCAGAGCTTAAAGAATTACTTTCAGATATATTAT TAAAAAGACATCCAGATGAAATTGATAAGATCAATCCTATTAAGAATTCAGCAAATGAAATTTCAAA ATTGAAGGAAGATCTTTCTCACCATCATCACCACCATTAG

Nucleotide sequence in bold are Shine-Dalgarno motif upstream of *L. crescens* genes. Italicized are spacer sequences. Underlined is the secretion signal from SDE1.


a
RE
Promoter
SD
Spacer
Full length GFP
RE
AGGAG
AGGAA
L. crescens codon optimized


b
L. crescens
culture
Collect cell
pellet
Resuspend pellet in
2xLaemmli buffer

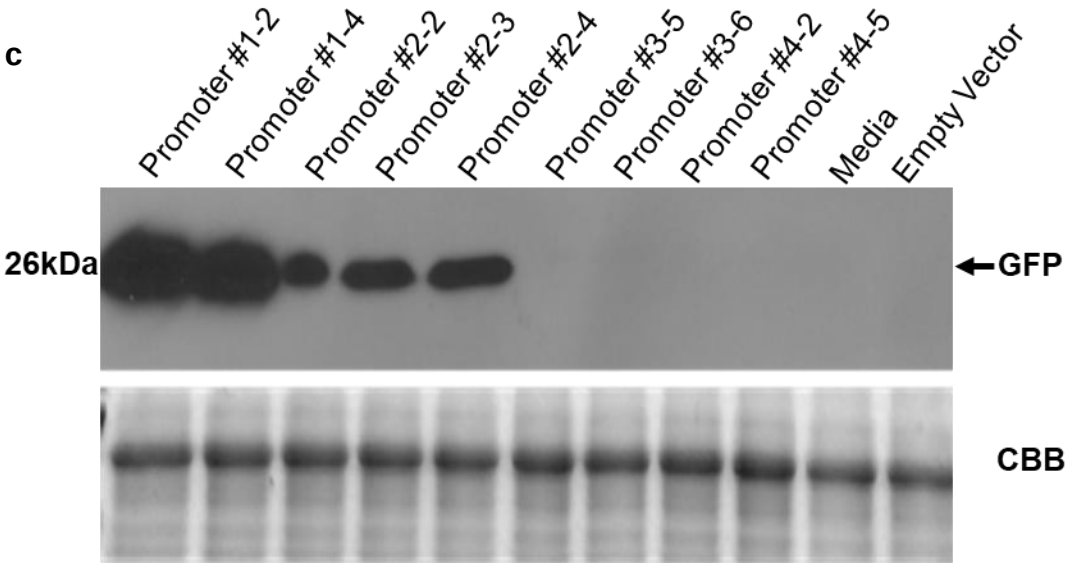

c
Promoter #1-2
Promoter #1-4
Promoter #2-2
Promoter #2-3
Promoter #2-4
Promoter #3-5
Promoter #3-6
Promoter #4-2
Promoter #4-5
Media
Empty Vector
26kDa
GFP
CBB

Figure 3.2. *Liberibacter crescens* cells express green fluorescent protein under Promoter #1 and #2. a) Diagram illustrating synthesis design for the four promoters, using restriction enzymes (RE), two Shine-Dalgarno (SD) sequences and a spacer before green fluorescent protein (GFP). Total protein was extracted from bacterial cells and expression was determined by Western blotting using anti-GFP antibody. **b)** Diagram illustrating preparation of samples **c)** Signal is detected from GFP expressed under promoters #1 and #2 but not from empty vector or promoters #3 and #4. Numbers indicate different plasmid transformations of the same promoter. Signal is detected at GFP's expected size of 26 kDa. Gel stained with Coomassie brilliant blue (CBB) served as loading control.

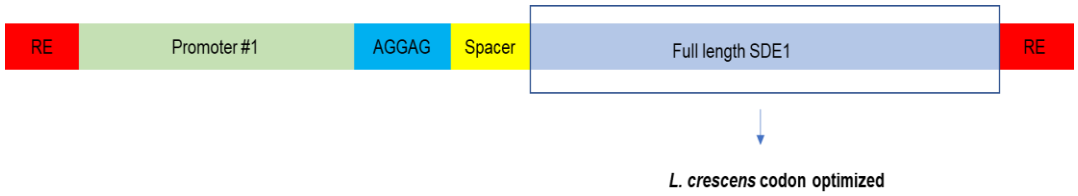
L. crescens expression of Sec-delivered effector 1 (SDE1)

I selected secreted effector SDE1 to test if our system will successfully express and secrete proteins cloned downstream of promoter #1 determining whether SDE1 secretion signal will be functional in *L. crescens*. Codon optimized, full-length *SDE1* was synthesized downstream of promoter #1 **(Table 3.1)**. I cloned these constructs into pUFR071 (Fleites et al., 2014) and transformed them into *L. crescens.* I confirmed protein expression of two *L. crescens* transformants carrying pUFR071::*SDE1*-9 or pUFR071::*SDE1*-10 then tested protein secretion. To test if SDE1 will be secreted, I grew both transformants in liquid culture and separated the cell pellet and cell-free supernatant using centrifugation. I precipitated the cell-free supernatant. Using Western blotting I detected protein expression in the pellet of pUFR071::SDE1-9 and pUFR071::*SDE1*-10 but not in the cell-free supernatant **(Figure 3.3)**. *L. crescens* carrying pUFR071::empty vector served as negative control and did not show signal in the Western blot.

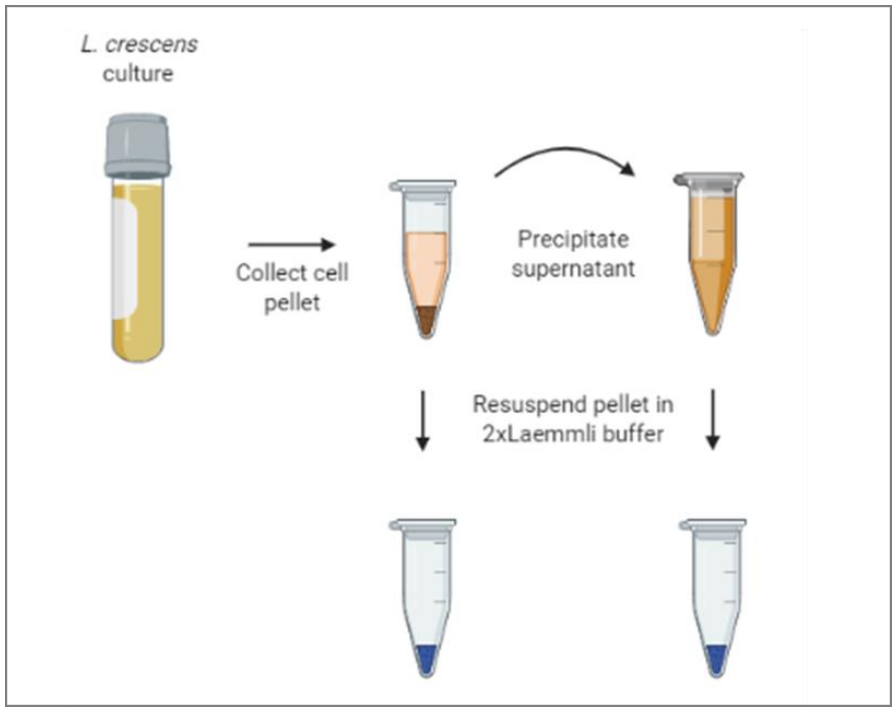
Sec secreted proteins travel to the periplasmic space where the secretion signal is cleaved. Given the fact I had not detected SDE1 expression in the supernatant, I presumed it's possible the proteins remained in the periplasm. I grew out *L. crescens* carrying pUFR071::*SDE1*-9 and pUFR071::*SDE1*-10 in liquid culture then collected cell pellet using centrifugation. I performed osmotic shock extraction on the cell-pellet to isolate the periplasmic proteins and precipitated them. However, I could not detect SDE1 protein in the periplasmic

portion **(Figure 3.4)** *L. crescens* carrying pUFR071 empty vector served as negative control and did not show signal in the Western blot.

a


RE
Promoter #1
AGGAG
Spacer
Full length SDE1
RE
L. crescens codon optimized

b


L. crescens culture
Collect cell pellet
Precipitate supernatant
Resuspend pellet in 2xLaemmli buffer

c

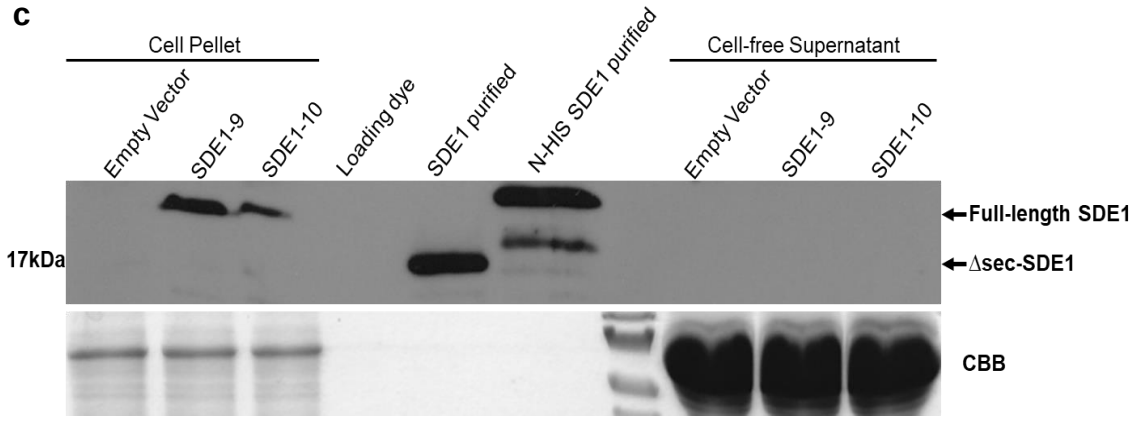

Cell Pellet
Cell-free Supernatant
Empty Vector
SDE1-9
SDE1-10
Loading dye
SDE1 purified
N-HIS SDE1 purified
Empty Vector
SDE1-9
SDE1-10
17kDa
Full-length SDE1
Δsec-SDE1
CBB

Figure 3.3. SDE1 detected in *Liberibacter crescens* cell pellet but not cell-free supernatant under Promoter #1. a) Diagram illustrating synthesis design for full-length SDE1 under Promoter #1 with Shine-Dalgarno (SD) sequence: AGGAG and restriction enzymes (RE). **b)** Diagram illustrating preparation of samples **c)** Total proteins from two *L. crescens* cultures (SDE1-9 and SDE1-10) were extracted from cell-pellet portion and cell-free supernatant which was additionally protein precipitated. Expression was determined by Western blotting using anti-SDE1 antibody. Signal is detected from bacterial pellets but not from supernatant indicating SDE1 was not secreted. Purified SDE1 protein without secretion signal (∆sec-SDE1) serves as size control for secreted SDE1. SDE1 purified using N-terminal 6xhistidine tag serves as size control for full-length SDE1 (before secretion). Signal is detected at approximately 20kDA which is slightly larger than full-length SDE1 expected size (18kDa). Gel stained with Coomassie brilliant blue (CBB) served as loading control.

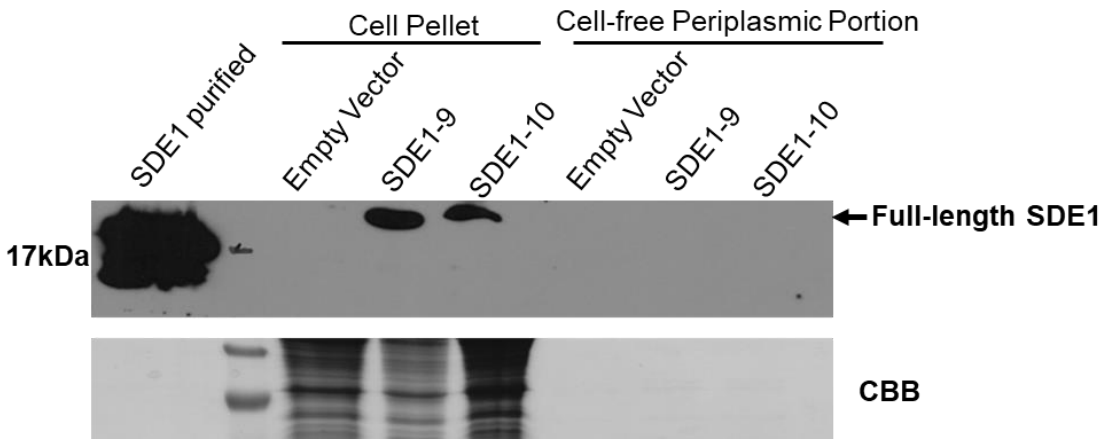
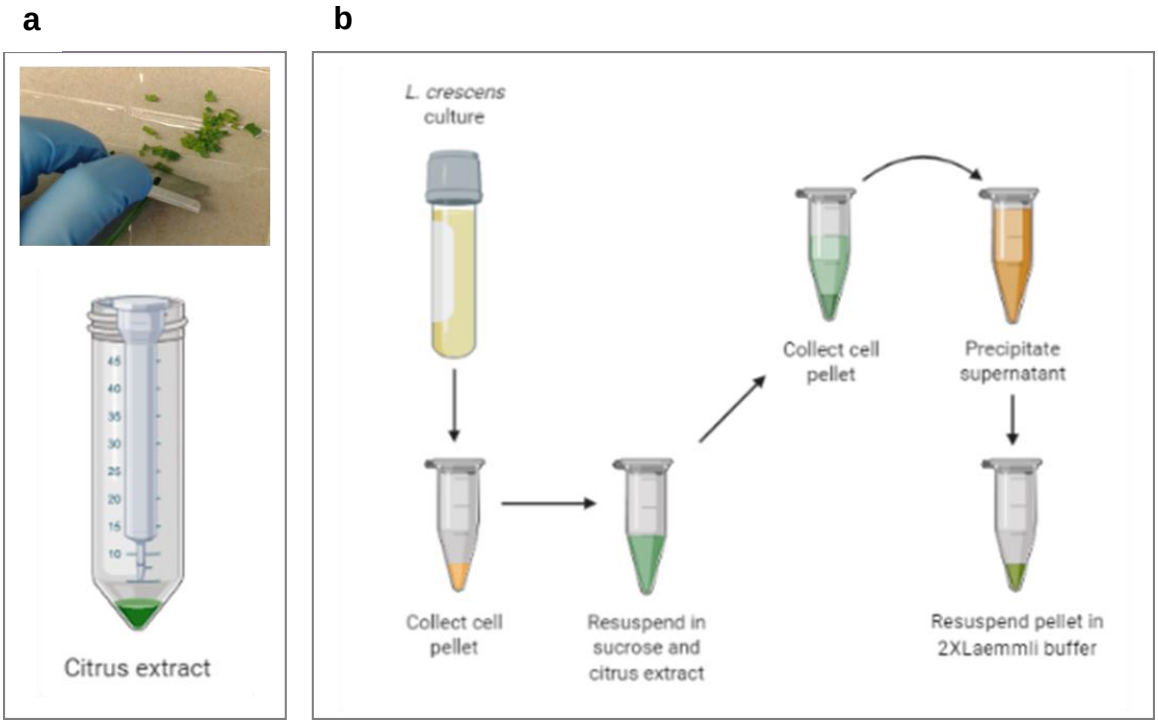


Figure 3.4. SDE1 detected in *Liberibacter crescens* cell pellet but not cell-free periplasmic portion under Promoter #1. Total proteins from two *L. crescens* cultures (SDE1-9 and SDE1-10) were extracted for cell-pellet portion while osmotic shock was used to isolate the periplasmic portion. Periplasmic samples were protein precipitated. Expression was determined by Western blotting using anti-SDE1 antibody. Signal is detected from cell-pellets but not from cell-free periplasmic portion indicating SDE1 was not secreted to the periplasm. Purified SDE1 protein without secretion signal serves as size control for secreted SDE1. Signal is detected at approximately 20kDA which is slightly larger than full-length SDE1 expected size (18kDa). Gel stained with Coomassie brilliant blue (CBB) served as loading control.

L. crescens secretion of Sec-delivered effector 1 (SDE1) is induced by citrus extract

Previous work in our lab on another citrus pathogen, *Spiroplasma citri*, informed us that providing plant extract to bacterial cells can induce proper induction of protein production (Shi et al., 2014). To explore whether SDE1 secretion by *L. crescens* can be plant induced, I mixed *L. crescens* bacterial carrying pUFR071::SDE1-9, pUFR071::*SDE1*-10, or pUCR071::EV with citrus extract and incubated the samples overnight at 28°C followed by protein precipitation. I detected a smaller protein size for the plant treated samples compared to cell pellet indicating the secretion signal was cleaved upon protein delivery outside of the cytoplasm **(Figure 3.5)**. As a control for protein size, I included purified SDE1 protein without secretion signal to mimic the protein size of plant treated/secreted SDE1 from *L. crescens* and the proteins are close in size.


a
b
Citrus extract
L. crescens culture
Collect cell pellet
Resuspend in sucrose and citrus extract
Collect cell pellet
Precipitate supernatant
Resuspend pellet in 2XLaemmli buffer

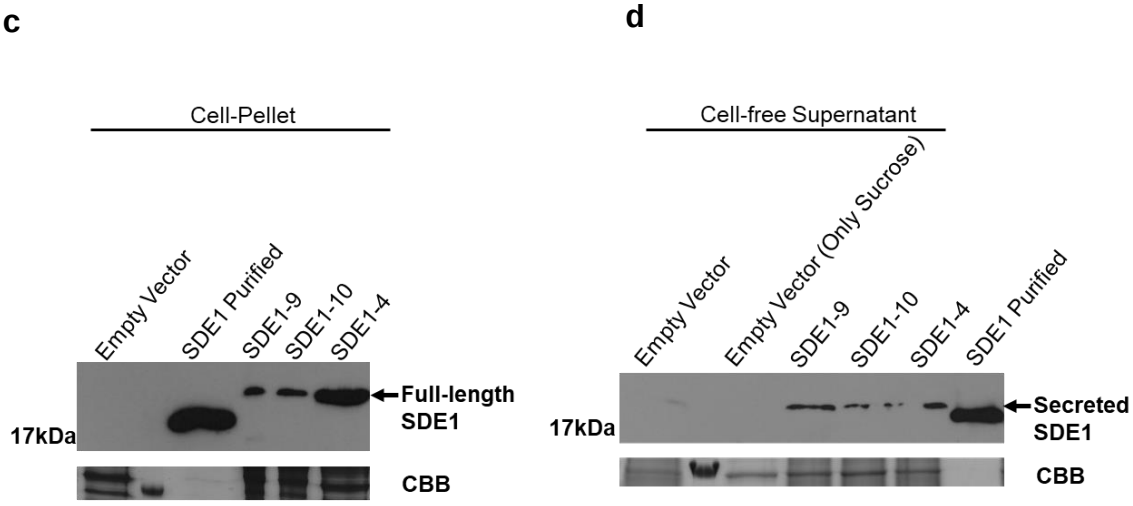

c
d
Cell-Pellet
Empty Vector
SDE1 Purified
SDE1-9
SDE1-10
SDE1-4
17kDa
Full-length SDE1
CBB
Cell-free Supernatant
Empty Vector
Empty Vector (Only Sucrose)
SDE1-9
SDE1-10
SDE1-4
SDE1 Purified
17kDa
Secreted SDE1
CBB

Figure 3.5. *Liberibacter crescens* secretion of SDE1 induced by citrus extract. a) Diagram illustrating preparation of citrus extract. **b)** Diagram illustrating *L. crescens* cell induction by citrus extract from Oroblanco grapefruit (*Citrus grandis* Osbeck x *C. Paradisi* Macf.) and protein precipitation. **c)** Total protein from three *L. crescens* cultures expressing SDE1 was extracted from cell-pellet portion. **d)** *L. crescens* pellets of the same three cultures were resuspended in sucrose and citrus extract then incubated overnight at 28°C followed by protein precipitation of cell-free supernatant. Purified SDE1 protein without secretion signal serves as size control for secreted SDE1. Expression was determined by Western blotting using anti-SDE1 antibody. Signal detected from supernatant is ~2Kda smaller than signal detected from cell pellets indicating SDE1 was secreted. Gel stained with Coomassie brilliant blue (CBB) served as loading control.

L. crescens screening tool for of Sec-delivered effectors

Ultimately the purpose for developing *L. crescens* as a screening system is to identify whether *C*Las predicted secreted effectors will be secreted in a close relative. Secretion prediction programs are dependent on the N-terminal secretion signal to identify proteins that are released outside of the bacterial cell. However, membrane lipoproteins which will be secreted outside of the cytoplasm but remain linked to the inner or outer membrane will also be predicted to be secreted. I chose CLIBASIA_00520 because it is predicted to be secreted but is also predicted to function as a lipoprotein indicated it is not released from the bacterial cell (lipoprotein screening using PROSITE software: https://prosite.expasy.org); therefore, it should not be detected in the *L. crescens* supernatant. Twist Bioscience synthesized codon optimized CLIBASIA_00520, with a C-terminal 6xHIS tag, downstream of promoter #1 **(Table 3.1)**. I cloned the synthesized fragment into pUFR071 then transformed it into *L. crescens*. *L. crescens* transformants expressing CLIBASIA_00520-6xHIS were treated with citrus extract and incubated overnight at 28°C followed by protein precipitation. I detected CLIBASIA_00520-6xHIS protein expression in the cell pellet but not the cell-free supernatant indicating it was not secreted outside of the cell **(Figure 3.6)**. *L. crescens* carrying pUFR071::empty vector served as negative control and did not show signal in the Western blot.

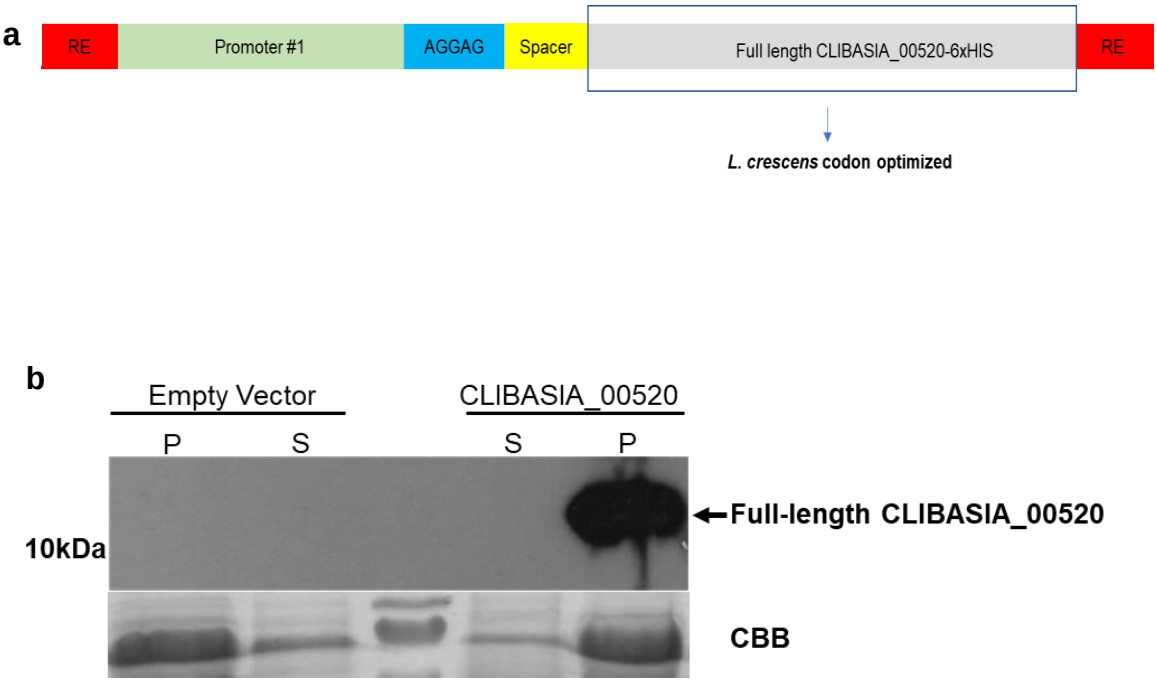


Figure 3.6. Citrus extract did not induce secretion of CLIBASIA_00520-6xHIS in *Liberibacter crescens*. a) Diagram illustrating synthesis design for CLIBASIA_00520 with a 6xHIS tag under Promoter #1, Shine-Dalgarno (SD) sequence: AGGAG and restriction enzymes (RE). **b)** Total proteins were extracted from *L. crescens* cell-pellet portion (P). *L. crescens* pellets of the same culture were resuspended in sucrose and citrus extract from Oroblanco grapefruit (*Citrus grandis* Osbeck x *C. Paradisi* Macf.) then incubated overnight at 28°C followed by protein precipitation of supernatant (S). Signal was detected by Western blotting using anti-HIS antibody at approximately the predicted size of CLIBASIA_00520-6xHIS (14kDa) in the cell-pellet portion but not cell-free supernatant indicating protein was not secreted.

DISCUSSION

The coevolution between plants and pathogens has culminated in large arsenals of immune receptors present in plant genomes that pathogens must disable in order to gain entry into the plant interior, colonize diverse tissues, and cause disease (Toruño et al., 2016). A critical component required for pathogenesis is the secretion of pathogen proteins, called effectors, which modulate plant immunity and facilitate infection (Macho and Zipfel, 2015). *C*Las lacks the type III secretion system (T3SS) found in many pathogenic gram-negative bacteria; however, it possesses the general Sec secretion system, which is capable of secreting effectors directly outside bacterial cells (Duan et al., 2009; Sugio et al., 2011). Sec-delivered effectors (SDEs) are among the most likely *C*Las virulence components (Pagliaccia et al., 2017; Clark et al., 2018; Thapa et al., 2020). Thapa and colleagues recently identified eight Sec-dependent SDEs effectors (including SDE1) present in *C*Las isolates which were all expressed at higher levels in citrus than in psyllids (Thapa et al., 2020). I confirmed software predictions by observing secretion of SDE1 but not of predicted lipoprotein CLIBASIA_00520. Using the system I've presented, effector secretion can be confirmed and depending on their predicted function, possibly validated as part of the bacterial pathogenic repertoire. Proteins can be expressed or possibly purified from *L. crescens* and tested for function *in vitro* or semi *in vitro*. For example, if a protein is predicted to be secreted and suspected to be a virulence factor or have certain enzymatic functions, then it can be

expressed in *L. crescens* and incubated with possible interactors involved in plant defense in a semi *in vitro* experiment where one protein is expressed in bacteria and the other *in planta*.

Significant effort has undergone to optimize detection of *C*Las infection in trees (discussed in Chapter 2). In order to produce antibodies for these serological based assays, an accurately expressed *C*Las protein serves as the antigen and upon injection into the mammalian rabbit system leads to the generation of anti-*C*Las protein antibodies that can be used in immuno-based detection assays. Expressing *C*Las proteins can be accomplished using the *L. crescens* expression system as opposed to *E. coli*, which is robust but may lead to protein misfolding affecting correct antigen production (Baneyx, 1999; Mergulhão et al., 2004). Currently, my research shows that citrus extract induces the secretion of effectors, but for purification purposes the protein (antigen) needs to be pure so we still need to test whether the antigen can be purified after mixing with citrus extract. If we identify key plant components that induce *L. crescens* secretion of SDE1, then we can synthesize these compounds and possibly induce protein section in a less contaminated manner. These components must not elicit an immunogenic response from the rabbit or the antibody will detect plant compounds. Interestingly, T3SS expression and delivery of T3SEs is significantly enhanced by different plant metabolites including citric acid, aspartic acid and shikimic acid (Anderson et al., 2014). Moreover, metabolites such as citrate, were also shown to be strong inducers of

coronatine production (a phytotoxin from the plant pathogen *Pseudomonas syringae*) (Li et al., 1998). There are examples that indicate a general role for these metabolites in activating different virulence mechanisms. It is possible there is a similar role for citrus metabolites in *L. crescens* protein secretion and this theory would greatly benefit from another plant that does not induce secretion, perhaps *Arabidopsis thaliana*, to serve as a control for transcriptomic changes occurring during incubation with citrus extract.

In this work SDE1 is under a constitutive promoter; therefore, it is transcribed and translated but protein secretion cannot be detected until incubation with citrus extract. In 2013, Yan et al. showed that there is an up-regulation of *C*Las Sec-associated genes in plants vs psyllids suggesting *L. crescens in vitro* culture may not have high enough expression of this component for me to detect protein expression (Yan et al., 2013). General hypotheses have been proposed regarding *C*Las delivery of Sec-delivered effectors, 1) *C*Las could be hijacking *flp* pilus assembly (functions similarly to the Type II secretion system) to secrete proteins extracellularly through the outer membrane beta-barrel porins (Johnson et al., 2006; Cong et al., 2012; Selkrig et al., 2012). 2) Secretion across the outer membrane is through Type I secretion which *C*Las harbors all the components for but its activity has not been well investigated (Duan et al., 2009; Cong et al., 2012). Finally, our understanding of how *L. crescens* is secreting proteins will improve the applications of this proxy system to enhance our grasp of *C*Las pathogenesis.

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GENERAL CONCLUSIONS AND DISCUSSION

Secreted effectors of bacteria are often employed to overcome plant defenses. Considering the global food crisis our society is predicted to face, we need to speed-up our work on plant pathogens. Studying one effector may elucidate a key pathogenic mechanism, allowing us to hinder its function and decrease a pathogen's virulence. However, typically one effector or toxin is used with an array of other effectors and we need to understand the cumulative function of the effector repertoire to alleviate the constraints these pathogens cause.

Studying a couple of effectors per project has provided a foundation that can be built upon to fulfill much larger screenings and experiments to bring our knowledge and materials together. For example, our work on HopZ1a mediated degradation of JAZ10 is one contribution, and other labs have worked on a couple of other JAZ's and have generated mutant lines that would allow us, as a scientific community, to further investigate utilizing these targets to withstand pathogen manipulation of plant hormones. Furthermore, multiple labs have screened predicted secreted effectors of *C*Las and by bringing the materials together and using *L. crescens* to deliver these effectors into a plant, perhaps in a collaborative effort, we can identify whether any known resistance proteins can recognize an effector. Generating a citrus plant with resistance proteins to *C*Las may limit the spread of the bacteria in the tree.