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A Novel Study of Interactions of *Arabidopsis thaliana* With *Candidatus* Liberibacter psyllaurous When Transmitted by psyllid *Bactericera cockerelli* to Develop a Chemical Genomics Based Approach that can Aid in the Development of Control Strategies for Huanglongbing Disease In Citrus.

A Dissertation submitted in partial satisfaction
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ABSTRACT OF THE DISSERTATION

A Novel Study of Interactions of *Arabidopsis thaliana* With *Candidatus* Liberibacter psyllaurous When transmitted by psyllid *Bactericera cockerelli* to Develop a Chemical Genomics Based Approach that can Aid in the Development of Control Strategies for Huanglongbing Disease in Citrus.

by

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Huanglongbing (HLB) is a bacterial disease associated with *Candidatus* Liberibacter asiaticus (CLas), *Candidatus* Liberibacter americanus, and *Candidatus* Liberibacter africanus. These bacterial species are transmitted by hemipteran psyllids. For many years, this disease has caused substantial damage to citrus around the world and in recent years, it has significantly impacted citrus production in the U.S.A. The lack of effective control and curative measures against psyllids and the pathogen are the major factors contributing to HLB-related damages. In addition to these factors, limitations such as longer generation time, limited genomics resources and regulatory restrictions hinder rapid research on HLB. Herein, I report a novel model plant-pathosystem of *Arabidopsis thaliana* and *Candidatus* Liberibacter psyllaurous (CLps) that has potential to facilitate rapid development of control strategies for HLB disease. Based on 98% identity in *16S rDNA* sequence and other genome sequence similarities, CLps is considered as a close relative to CLas. The CLps pathogen can infect solanaceous plants

such as tomato, potato and pepper. To our knowledge, this is the first study demonstrating Liberibacter infection in *Arabidopsis*. The CLps pathogen can be transmitted by psyllid *Bactericera cockerelli* (BC) into *Arabidopsis*. Over time an increase in bacterial titer was evident in 19 genetically diverse ecotypes and defense-compromised lines of *Arabidopsis.* Similar to CLas, CLps systemically infects *Arabidopsis.* I identified several similarities between the phenotypic and transcriptional changes in CLps infected Col-0 and *NahG* (a salicylic acid deficient Col-0) lines and the responses in CLas infected citrus. Some unique changes such as development-related transcriptional changes associated with floral transition and a novel phenotype of excessive growth of small size axillary/cauline leaves were observed only in CLps infected *Arabidopsis.* A foliar spray technique for testing candidate defense-inducing chemicals with *Arabidopsis*/BC/CLps system and detached leaf assay with tomato/BC/ CLps system were evaluated for their effects on Liberibacter infection. The timeline of experimental assays with *Arabidopsis*–CLps is considerably shorter than that for HLB-citrus related assays reported previously. Our model plant-pathosystem can be used as a resource in HLB-related preliminary studies to more rapidly understand the effect of (or manipulate) potential genes and defense inducing chemicals on Liberibacter infection.

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GENERAL INTRODUCTION

Citrus Industry and diseases

Citrus fruits are among the most widely grown and consumed fruit crops worldwide. Originated in Asia, these fruits are grown in more than 100 countries with tropical, subtropical and Mediterranean climate conditions. Compared with other species of *Citrus*, oranges (61%) and tangerines / mandarins (22%) form the largest portion of citrus fruit produced in the world (www.fao.org). Brazil, China and the USA are the leading growers of oranges producing about 62% of world production, most of which is used for juice (Foreign Agricultural Service (FAS),USDA, 2014). In the USA, *Citrus* fruits are grown over 797 thousand acres producing 11,000,000 tons of fruits with a production value of $ 3.15 billion (NASS 2013). Florida, California, Texas and Arizona contribute to the majority of citrus production in the U.S.A (National Research Council, 2010). Of the total fruit production, 96% is produced in Florida and California (National Research Council, 2010). The economic contribution of the citrus industry is often threatened by different biotic (pre and post harvest diseases) and abiotic (hurricanes, freezing temperatures, rainfall) challenges. In 2013, USDA released a report showing a continuous decline in the orange producing acreage and its associated value of production since 2004-05 (NASS, 2013). Such a decline in citrus growing acreage mostly results from the impact of diseases such as Huanglongbing (HLB), citrus canker and tristeza. Citrus canker disease is an exotic bacterial disease introduced to Florida around

1912. It can affect fruit quality and quantity of almost all the *Citrus* cultivars (Gottwald et al., 2002) and with no cure available for this disease, management using copper sprays and quarantines are the only control measures (Gottwald et al., 2002). Citrus tristeza is one of the most destructive viral diseases of *Citrus* and citrus tristeza virus (CTV) infects all the *Citrus* cultivars (Bar-Joseph et al., 1989). When infected with severe CTV isolates trees have poor quality fruits, dieback of stems and gradually die, whereas other CTV isolates can cause a quick decline of trees grafted on sour orange (Brlansky et al., 2014a). Both Tristeza and Canker have caused major damages to the *Citrus* industry; however in 2005 the discovery HLB infected trees in Florida was a detrimental addition to this group of diseases (Sutton et al., 2005).

Huanglongbing: *("Huang" means yellow, "long" means dragon "bing" means disease).* HLB is a one of the oldest and deadliest *Citrus* tree diseases. It is associated with the phloem-limited bacteria *Candidatus* Liberibacter species are transmitted into plants by insect (hemipteren) vectors known as *Citrus* psyllids. The initial reports of HLB disease date back to 1919 in southern China but the first report of the infectious nature of HLB was published in 1956 by Lin Kung Hsiang (Bové, 2006). HLB has been prevalent in many Asian and African countries for about 100 years. It was referred to by different names such as "yellow branch", "blotchy mottle" or "greening" in Africa, yellow shoot disease in Philippines, "likubin" in Taiwan, "dieback" in India, "leaf mottle" in Philippines and "vein phloem degeneration" in Indonesia based on disease symptomology (Bové

and Saglio, 1974; Chen et al., 1971; Laflèche and Bové, 1970b, 1970a; Tanaka and Doi, 1973). Huanglongbing is now considered as an official name for the disease (Moreno et al., 1996). The first reports of HLB in the western hemisphere were from Sao Paulo, Brazil (2004) and Florida, USA (2005), which placed two of the world's largest *Citrus* industries at risk (Sutton et al., 2005; Texeira et al., 2005). HLB disease is associated with the gram negative, sieve tube inhabiting *Candidatus* Liberibacter species. So far, three strains of HLB pathogens have been identified and named as *Candidatus* Liberibacter *asiaticus* (CLas) (Jagoueix et al., 1994), *Candidatus* Liberibacter americanus (CLam) (Texeira et al., 2005) and *Candidatus* Liberibacter africanus (CLaf) (Jagoueix et al., 1994). *Candidatus* Liberibacter asiaticus is the more widely spread and more severe strain compared to the African and American forms. It is heat tolerant and can induce disease symptoms at temperatures of 35°C (Batool et al., 2007; Garnier and Bové, 1993). At present, CLaf is geographically restricted to the African continent and CLam is found only in Brazil. By contrast, CLas is found in American and Asian continents. The CLas and CLam species are vectored by *Diaphorina citri* (Kuwayama) (Capoor et al., 1967; Texeira et al., 2005) also known as Asian Citrus psyllid (ACP) whereas the CLaf is vectored by *Trioza erytreae* Del Guercio (McClean and Oberholzer, 1965) also known as African Citrus psyllid (Catling, 1970). Depending on the temperature conditions, an ACP can complete all its life stages (eggs, nymphs and adults) in 15-47 days on *Citrus* (Catling, 1970).The ACP and HLB associated pathogens can infect nearly all the *Citrus* cultivars including mandarins, limes, lemons, sweet oranges, and pummelos as well as close

relatives of *Citrus* (Bhagabati, 1993; Halbert and Manjunath, 2004; Miyakawa, 1980; Miyakawa and Yuan, 1990). Depending on the tree age, its physiological state, and distribution of pathogen within the tree, trees exhibit varying degrees of symptoms. Yellowing or blotchy mottle appearance of leaves that resemble to zinc deficiency symptoms is one of the first symptoms to appear on infected trees (McClean and Schwarz, 1970). However, unlike zinc deficiency symptoms, leaves from HLB infected trees display asymmetric mottle distribution and corky veins. Twigs tend to dieback and foliage become sparse with an increase in severity of infection (Da Graca, 1991). Fruits developed on HLB-infected trees are small in size and are lopsided in shape (Da Graca, 1991). Juice from fruits of HLB infected trees have higher acidity, lower Brix/acidity (Bassanezi et al., 2009), and often taste bitter making it inedible. Young fruits tend to drop prematurely and mature fruits often remain green at the stylar end (Da Graca, 1991). Anatomical studies of HLB infected leaves showed phloem blockage caused by callose and starch accumulation most likely occurring as the plant responses to the HLB pathogen (Achor et al., 2010; Etxeberria et al., 2009). Such blockages can restrict nutrient flow between plant source and sink tissues and consequently result in a decreased productivity. So far, there are no efficient measures available to cure the HLB infected *Citrus* trees, however several research strategies have been pursued to prevent the spread of disease and maintain plant productivity. Control strategies include controlling the spread of psyllids by insecticide sprays, regulating the movement of *Citrus* bud wood by quarantine implementation, thermotherapy (Hoffman et al., 2013)

and application of antibiotics or other effective chemicals (Puttamuk et al., 2014) for pathogen control in plants.

Psyllid yellows, a solanaceous disease similar to HLB.

Psyllid yellows (PY) refers to a yellowing condition of tomato and potato plants caused by feeding of the psyllid *Bactericera cockerelli* (BC) (Blood, 1933b; Blood et al., 1933a; Richards, 1928, 1931). Until recently, PY was believed to be caused by a toxin injected by BC (Blood, 1933b), however Hansen et al. (2008) showed its association with the phloem limited bacterial species known as *Candidatus* Liberibacter psyllaurous (CLps, alternatively known as *Candidatus* Liberibacter solanacearum (CLso)) transmitted into plants by BC. Like the HLB pathogen, CLps is a gram-negative sieve tube inhabiting bacterium. Hansen's study (Hansen et al., 2008) showed its transmission into BC transovarially and by feeding on CLps-infected plants. BC is a polyphagous psyllid that can multiply on several solanaceous plant species such as *Solanum* lycopersicum (tomato), *Solanum tuberosum* (potato), and *Capsicum annum* (pepper) (Wallis, 1951). The life cycle of BC lasts for approximately 33 days and includes three life-stages, egg, nymph and adult. The timeline of different developmental stages of BC is dependent on host and temperature conditions (Liu and Trumble, 2007). A female psyllid lays ~500 eggs in its life which hatch within 6-7 days (Lehman, 1930). Eggs are yellowish orange in color and are usually seen along leaf edges (Lehman, 1930). The nymphal stage of this psyllid includes five instar stages lasting approximately 21-30 days. Third, fourth and

fifth instar stage nymphs can be differentiated by their light yellowish orange or greenish appearance. The adult psyllid is approximately 2.5mm-4mm from head to wing tip and has a banded grey appearance (Lehman, 1930). PY is a systemic disease and an entire plant can display disease related symptoms. The CLps-infected tomato plants display symptoms such as leaf yellowing or purpling of leaves, growth retardation, smaller size foliage, enlarged and shortened internodes, and early senescence leading to premature death (Cranshaw, 1994; Pletsch, 1947). Histological studies (Eyer, 1937) of diseased plants reveal phloem necrosis, distortion of chloroplasts and protein breakdown. Such structural damage can cause alteration in the carbohydrate metabolism and translocation processes via phloem tissues. Biochemical studies also show increased sucrose and carotene levels and reduced levels of chlorophyll (Eyer, 1937). The gene expression studies of tomato plants showed a complex and interesting pattern of defense gene induction in response to psyllids and CLps infection (Casteel et al., 2012). Transcripts encoding PR1 (PATHOGENESIS RELATED-1, Salicylic acid (SA) responsive gene) and AOS (ALLENE OXIDE SYNTHASE, Jasmonic acid (JA) responsive gene) were suppressed after graft or adult psyllid inoculation of CLps. However, several SA and JA associated mRNAs encoding PHENYLYALANINE LYASE 5 (PAL5), PROTEINASE INHIBITOR (PIN2), PR1 and AOS increased after CLps inoculation by younger psyllids. This data suggests that the plant defense mechanisms can be manipulated by CLps inoculum titer in psyllids.

Candidatus Liberibacter species

Candidatus Liberibacter (Liber (bark) bacter (bacteria)) species are gram-negative alpha-proteobacteria. These bacteria are referred by *Candidatus* status, a provisional status given to the prokaryote associated with, when it has not been cultivated in cultures, but its association with disease has been verified by other techniques such as amplification by sequence specific primers (Murray and Schleifer, 1994; Murray and Stackebrandt, 1995). Various *Candidatus* Liberibacter species have been identified and associated with plant diseases such Huanglongbing, Psyllid yellows and Zebra chip (Hansen et al., 2008; Jagoueix et al., 1994; Liefting et al., 2009; Munyaneza et al., 2009). CLps or CLso are known to infect solanaceous species such as tomato (*Solanum lycopersicon*), potato (*Solanum tuberosum*), pepper (*Capsicum annuum*), eggplant (*Solanum melongena* L.), silverleaf nightshade (*Solanum elaeagnifolium* Cav.), black nightshade (*Solanum ptychanthum* Dun.), wolfberry (*Lycium barbarum*), and *Apium graveolens* (Hansen et al., 2008; Munyaneza et al., 2007a, 2009; Teresani et al., 2014; Wen et al., 2009). Genomes of various Liberibacter species have been sequenced and sequence analyses revealed some interesting aspects of this genus (Duan et al., 2009; Lin et al., 2011; Wulff et al., 2014). Sequences of *Candidatus* Liberibacter species show close relation to the Rhizobiales but the genomes of *Candidatus* Liberibacter asiaticus and *Candidatus* Liberibacter americanus are significantly reduced compared to Rhizobium and Sinorhizobium (Duan et al., 2009; Lin et al., 2011; Wulff et al., 2014). All

three species, CLas, CLps and CLam, have sequences for flagellar proteins but electron micrographs of these bacteria (CLas) in plants do not show these structures (Bove, 2006). Comparative gene expression analysis of CLas infection in plants and psyllids reveal lower expression of the flagellin protein in planta than psyllid (Yan et al., 2013). . This indicates bacteria's avoidance of triggering the plant defenses (Yan et al., 2013). Genomes of CLas and CLso/CLps have a limited set of amino acid biosynthesis genes, however the presence of amino acid ABC transporters indicate that they depend on host cells amino acids (Lin et al., 2011). Evidence of ATP/ADP translocase protein indicates that CLas and CLso can take ATP (energy) from host cells (Duan et al., 2009; Lin et al., 2011). Some genes identified were serine/threonine and tyrosine phosphatase and metalloprotease and their predicted protein functions are suggested to be suppression of host plant signaling and defenses (Cong et al., 2012; Yan et al., 2013). Based on such genome sequence similarities between various *Candidatus* Liberibacter species, there is a possibility that similarities might also exist in their interaction with host plants. Based on this assumption, the development of a cure or preventative measures against any of these species can aid control diseases like HLB, PY and zebra chip. In this study, I investigated the potential of an approach known as chemical genomics that involves screening chemical compounds to induce desired effect with an objective of developing a control strategy for these pathogens.

Chemical Genomics:

Schreiber and Mitchison recognized chemical genetics as an approach with a potential to dissect gene or protein functions in desired biological processes using small molecular weight, and cell permeable chemicals (Mitchison, 1994; Schreiber et al., 2008). Chemical genomics is a similar approach that uses genome-scale data to understand gene and protein functions in desired biological processes. This approach uses a wide range of small molecules generated by combinatorial-based chemistry to probe biological questions. These small molecules can typically alter protein functions in a reversible, conditional and rapid manner, thereby modulating the biological processes. To identify a small molecule as a potential bioactive compound, one may determine the compound's ability to permeate membranes by Lipinski rule of five. Lipinski "rule of five" states that a small molecule may have poor absorption or permeation if its molecular weight is more than 500, if its lipophilicity (CLogP) is greater than 5, and when there are more than 5 hydrogen bond donors and 10 H bond acceptors in it (Lipinski et al., 1997). Identifying chemicals that have desired effects require screening of thousands of chemical compounds. Chemical libraries are typically synthesized in two types, diversity-oriented and focused type (Young and Ge, 2004). Diversity oriented chemical libraries contain chemical compounds that can modulate different classes of proteins and focused libraries contain compounds designed around a specific scaffold that interacts with a specific or related proteins (Stockwell, 2004). Typically, the

development of a chemical genomics approach for answering a biological question would involve the following 3 stages (Blackwell and Zhao, 2003) 1) develop a high throughput chemical screening assay with the desired organisms, 2) conduct a primary screen of chemicals to identify the chemicals producing desired effects and a secondary screen to validate candidate chemicals from primary screen, and 3) understand effect of the chemicals by phenotype characterization and target identification. Lethality and redundancy are often the major bottlenecks while pursuing mutational analysis approach to study processes in plants. Use of small molecules can overcome these challenges by altering closely related proteins simultaneously and modulating them in a conditional and reversible manner, thereby producing effects at a desired level and time point (Raikhel and Pirrung, 2005). Several studies demonstrate successful application of chemical genomics in plant developmental and defense-related research. A high throughput style chemical screen of 10,000 compounds resulted in the identification of 34 potential compounds affecting plant gravitopism (Surpin et al., 2005). Among the 34 compounds, one compound named as gravicin proved helpful in understanding auxin effects on vacuole morphology and endomembrane trafficking (Surpin et al., 2005). A screen of 10,000 compounds lead to identification of the two compounds with an ability to alter auxin related responses in plants (Armstrong et al., 2004). From a screen of 20,000 compounds library, a chemical compound named Morlin created a unique swollen root phenotype in *Arabidopsis* which proved useful to understand the mechanism of cortical microtubule organization (DeBolt et al., 2007). Several chemical

compounds have been identified that altered various hormone-signaling pathways. A compound known as 2-4 D Proauxin, a synthetic auxin, was identified that altered auxin related responses (Savaldi-Goldstein et al., 2008). It diffuses efficiently through tissues, is cleaved into functional auxins and enhanced hypocotyl growth in *Arabidopsis* (Savaldi-Goldstein et al., 2008). The advantage of chemical genomics over mutational analysis was evident in the study of GLYCOGEN SYNTHASE KINASE 3 (GSK3), an important component of the Brassinosteriod signaling pathway (De Rybel et al., 2009a). A compound named bikinin was identified that activated BR signaling by binding to various GSK3/BR-INSENSITIVE2 (BIN2) proteins (De Rybel et al., 2009a). These results showed that simultaneous inhibition of seven GSK3 proteins by bikinin was sufficient to activate BR signaling (De Rybel et al., 2009a), which otherwise was not easily possible with a mutational approach due genetic redundancy. A similar example of overcoming genetic redundancy by chemical genomics was demonstrated by identification of 12 ABA receptors (PYR/PYL) using pyrabactin, a growth inhibitor and selective ABA agonist (Park et al., 2009). Chemical genomics has also been successfully used in various plant defense related studies (Knoth et al., 2009; Schreiber et al., 2008; Serrano et al., 2007). Among a screen of 120 bioactive compounds, four compounds (triclosan, fluazinam, cantharidin, and fenpiclonil) were identified that inhibited the PAMP triggered or FLAGELLIN22 (FLG22) triggered responses in *Arabidopsis* seedlings (Serrano et al., 2007). Among these compounds triclosan inhibited the flg22 dependent induction of reactive oxygen species and this compound was further used to understand the role of lipid

signaling in flg22-mediated early defense responses (Serrano et al., 2007). From 3000-member LATCA library, a compound named Sulfamethoxazole (SMEX) was identified that reduced *Pseudomonas syringae* bacterial growth in *Arabidopsis* seedlings (Schreiber et al., 2008). Knoth et al. (2009) demonstrated that a chemical compound 3,5-Dichloroanthranilic acid (DCA) acts as a synthetic defense inducer capable of triggering defenses in *Arabidopsis thaliana* in response to *Hyalopernospora parasitica* and *Pseudomonas syringae*. Most of these chemical genomics-related plant studies based on a high throughput screening of chemical compounds with *Arabidopsis thaliana*. To use the advantage of numerous resources available for *Arabidopsis thaliana,* especially in relation chemical genetics, we investigated the suitability and potential of *Arabidopsis* for CLps infection and conducting chemical screening.

***Arabidopsis thaliana* as a model plant system to study plant-pathogen interactions**

Understanding the molecular basis of any disease is important for designing an effective cure for the disease. However, genetic and biochemical characterization of plant-pathogen interactions is often hindered by the lack of information or lack of effective techniques. These challenges are heightened when genetic variability of the plant species adds complexity to the responses to pathogens. In such cases, it could be useful to study plant species whose molecular mechanisms are well studied and tractable to enable a systematic dissection of molecular mechanisms. *Arabidopsis thaliana* is an annual, dicotyledonous plant belonging to family *Brassicaceae* that meets many of these

requirements. It is a small size plant with a 125 Mb genome and a rapid life cycle of 6-8 weeks from seed germination to seed production. Ease of manipulation and replication provides a significant advantage to *Arabidopsis* over other plant species. Availability of a full sequenced genome (The *Arabidopsis* Genome Initiative, 2000), extensive genetic and genomic resources such as genetic and physical maps of all chromosomes and numerous mutants and transgenic lines makes *Arabidopsis* highly useful in plant research. It has been widely used in many developmental and defense related studies as a model plant to elucidate gene functions and processes. *Arabidopsis* has been tested with different microorganisms to develop model plant-pathosystems (Davis and Hammerschmidt, 1993; Mauch-Mani and Slusarenko, 1993). Several studies demonstrate that *Arabidopsis* exhibits responses similar to other hosts to many pathogens (Ausubel et al., 1991; Bent et al., 1991; Dangl et al., 1991; Daniels et al., 1991; Debener et al., 1991; Tsuji et al., 1991). Plants showed increased resistance to bacterial wilt and Fusarium wilt in tomato and Fusarium blight in wheat upon genetic transformation with the *NPR1* gene of *Arabidopsis* (Lin et al., 2004; Makandar et al., 2006). *Arabidopsis thaliana* has been used in many *Citrus* related defense studies to dissect the role of defense genes and to develop disease resistant varieties (An and Mou, 2012; Lu et al., 2013; Zhang et al., 2010). NON-RACE SPECIFIC DISEASE RESISTANCE 1 (NDR1) is a plasma membrane localized protein and plays an important role in the signal transduction of coiled coil (CC) nucleotide-binding site (NBS) and leucine-rich repeat (LRR) resistance genes (Century et al., 1995, 1997; Peter Coppinger et al., 2004).

Mutations in *NDR1* gene resulted in its loss of function and rendered *Arabidopsis thaliana* (*ndr1-1*) susceptible to *Pseudomonas syringae* expressing the avirulence effector *avrRpt2* (Century et al., 1995). In order to dissect the role of *Citrus NDR1* (CsNDR1) gene, *Arabidopsis ndr1-1* mutant was complemented with *CsNDR1* (Lu et al., 2013). The overexpression of *CsNDR1* lead to increased resistance in *Arabidopsis* to bacteria *P.syringae.avrRpt2* as well as to the oomycete pathogen *Hyaloperonospora arabidopsidis* isolate Noco2. *NON-EXPRESSER OF PATHOGENESIS RELATED 1* (*NPR1*) is a crucial regulator of the SA pathway and systemic acquired resistance (SAR). Studies show that induction of SAR can be effective against *Citrus* canker disease (Francis et al., 2009). Overexpression of *Arabidopsis AtNPR1* in Hamlin orange and Duncan grapefruit resulted in a ten-fold reduction of lesions formed by the pathogen (Zhang et al., 2010). Study of *Arabidopsis* and its interaction with *Xanthomonas citri subsp. citri*, a non-host pathosystem, revealed that genetic components of the SA pathway play a crucial role its non-host defense responses (An and Mou, 2012). Such studies demonstrate the potential use of *Arabidopsis* to understand novel plant-pathogen interactions.

Dissertation project:

My dissertation study was initiated with a goal to identify chemical compounds that can elicit defenses in model plants like tomato and *Arabidopsis* against *Candidatus* Liberibacter psyllaurous and potentially to HLB pathogens. The key requirement for this objective was a suitable plant-pathosystem that can be tractable and optimum to

conduct high-throughput chemical screening. We studied responses of *Arabidopsis* to CLps infection at the phenotype and molecular levels to determine its potential as model plant-pathosystem. In addition to studying responses, different chemical screening assays were evaluated with this plant-pathosystem to test chemicals likely to induce defenses. In Chapter 1, I report the first evidence of transmission of *Candidatus* Liberibacter by *Bactericera cockerelli* and symptoms resulting from CLps infection in genetically diverse ecotypes and defense-compromised lines of *Arabidopsis thaliana*. In Chapter 2, a detailed transcriptional and phenotypic analysis of *Arabidopsis'* responses to CLps infection is presented. In this study, two lines the Columbia-0 (Col-0) ecotype and *NahG,* (Col-0 transformed with transgene salicylate hydroxylase) were used to study the responses. *NahG* line is deficient in salicylic acid (SA), however had a similar percentage of CLps positive plants to Col-0 (Chapter 1). This line was used to determine the effect of reduced SA levels on *Arabidopsis* responses to CLps infection via psyllid transmission. These responses were studied at two times; 3 weeks post infestation (wpi, a separate experiment, only with candidate genes), and 8 wpi (Microarray experiment). The phenotypic changes from 3wpi to 8wpi in response to CLps infection are also reported in Chapter 2. In my last chapter (Chapter 3), I present results of testing various chemical-screening assays such as detached leaf assay and foliar sprays to study chemical absorption into plants and their effect on CLps infection.

CHAPTER 1: A novel investigation of responses of *Arabidopsis thaliana* to *Bactericera cockerelli* transmitted *Candidatus* Liberibacter psyllaurous as a model vector-pathogen system for Huanglongbing disease.

SUMMARY:

Many research efforts have been undertaken to prevent the spread of Huanglongbing (HLB) or to develop a cure for it. However, challenges associated with citrus species such as longer generation time, limited genomic resources and quarantine regulations for HLB (vector and pathogen) often hinder fast and effective research. A tractable model plant-pathogen system can provide significant advantages with respect to time and resources in comparison to citrus-HLB system. Research studies with citrus canker provide evidence that *Arabidopsis thaliana* can serve as model plant system for a use in understanding mechanism of citrus diseases. Herein, we used *Candidatus* Liberibacter psyllaurous (CLps, pathogen associated with psyllid yellows), a close relative of the HLB pathogen. The CLps bacteria are transmitted by the psyllid *Bactericera cockerelli* into solanaceous plants. Comparison of CLps and HLB pathogens reveal several similarities in their bacterial genome sequences, transmission mechanisms and symptomology indicating the potential of BC/CLps as a model pest-pathogen system for HLB pathosystem. With availability of well-established protocols, numerous genomic resources and features such as high seed count and short life cycle, *Arabidopsis thaliana* can serve as an effective and tractable tool to understand CLps-triggered plant

responses. In an effort to understand the potential of *Arabidopsis thaliana* as a model plant system, nineteen genetically diverse *Arabidopsis* ecotypes were investigated for their responses to CLps infection resulting from the feeding of CLps positive psyllids. In the preliminary screen, all ecotypes had CLps infected plants, albeit with a range of 20%-100% for different ecotypes and variable bacteria titers in different tissues. To validate these preliminary screen results, a repeat experiment with nine ecotypes was conducted with more replicates. However, the responses to bacterial infection among ecotypes were not consistent with our preliminary screen. In both ecotype-screening experiments, no distinct disease related symptoms were observed in any CLps positive plants at 5 weeks post infestation (wpi). To test the hypothesis that *Arabidopsis thaliana* is tolerant to CLps and may not display any CLps-related symptoms, five defense mutant lines compromised in either salicylic acid or jasmonic acid or ethylene pathways were tested for an extended time post psyllid feeding. Similar to the ecotype screening results, a variable proportion of plants of defense mutant lines were detected positive for CLps infection. Interestingly, at 6 wpi to 7 wpi several plants of defense compromised lines displayed distinct disease symptoms including discoloration of leaves (purple leaves), excessive growth of small, new axillary leaves from the rosette center, lack of stem or smaller stems with curled leaves. Wild type Col-0, *npr1-1* (Salicylic acid mutant), *NahG* (Salicylic acid deficient), *ein2-5* (Ethylene insensitive) and *coi-1* (Coronatine insensitive-1) had high numbers of CLps positive plants that distinctly showed CLps related symptoms compared to other lines.

ABBREVIATIONS:

HLB- Huanglongbing, CLps- *Candidatus* Liberibacter psyllaurous, CLas- *Candidatus* Liberibacter asiaticus, SA: Salicylic acid, ET: Ethylene, JA: Jasmonic acid, SAR: Systemic Acquired Resistance, *PR1*: pathogenesis related 1, wpi: weeks post infestation, BC: *Bactericera cockerelli*

INTRODUCTION

Plants constantly come in contact with various microorganisms, often non-pathogenic and infrequently pathogenic. Although, innate (preformed) defenses provide a crucial non-host defense to plants, at times these plants fail to fight evolutionarily advanced pathogens due to lack of genetic components essential for triggering adequate defenses. Such scenarios can have disastrous effects on our food supply if the targeted plants are economically important in agriculture. Citrus industry is one of the largest fruit crop industries in the world. Diseases such as Huanglongbing (HLB), Tristeza and Canker are some of the destructive diseases that have caused billions of dollars losses to citrus industry. Due to the lack of cure and effective control measures, HLB is considered as one of the most destructive diseases in the citrus industry. Several research efforts on screening genetically diverse varieties of *Citrus* and its close relatives are underway to identify HLB resistant species. Research on different aspects of plant-pathogen interactions is also necessary for development of control strategies for the disease. However, characteristic features of citrus such as a long generation time, self-incompatibility, apomixis and limited availability of genomics tools delay rapid development of such strategies for citrus diseases.

Huanglongbing:

HLB is associated with three phloem limited bacterial species *Candidatus* Liberibacter asiaticus (CLas) (Jagoueix et al., 1994), *Candidatus* Liberibacter americanus (CLam) (Texeira et al., 2005), and *Candidatus* Liberibacter africanus (CLaf) (Jagoueix et al., 1994). These bacteria are transmitted into trees by hemipteren psyllids known as *Diaphorina citri* Kuwayama (Capoor et al., 1967; Texeira et al., 2005) and *Trioza erytreae* Del Guercio (McClean and Oberholzer 1965). Grafting of HLB-infected budwood on healthy citrus trees can also spread bacteria into healthy groves. After the transmission of bacteria into a tree, there is a latent period when the tree remains asymptomatic. Depending on the variety, age and location, symptoms can appear between 3-6 months to a year (Halbert and Manjunath, 2004). As the pathogen titer increases, plants develop symptoms such as yellowing of leaves or leaves with blotchy mottle appearance. In severely infected trees, leaves drop, twigs tend to dieback, and fruits remain green in color and smaller in size with bitter fruit juices (Da Graca, 1991). Eventually, health severely deteriorates and the tree ultimately dies. Researchers have applied different approaches such as antibiotic injections (Puttamuk et al., 2014), and thermotherapy (Hoffman et al., 2013) to eliminate the pathogen from citrus seedlings. Although successful to a certain extent, these solutions are not completely efficient and effective in eliminating CLas in older trees and under all temperature/field conditions. There are no efficient preventative measures available for the HLB pathogen, which puts disease

free citrus growing areas at a huge risk. It is critical to study the interactions of plant and HLB pathogen at molecular level to design a rapid and robust strategy that can either eliminate or control the spread of CLas or reduce its damage to trees. However, the study of interactions of citrus trees with HLB pathogens has been slow and challenging primarily due to lack of in-vitro CLas cultures and failure to detect CLas efficiently due to its uneven distribution in trees. In our studies we investigate the use of psyllid yellows as potential model disease system that can provide rapid understanding of such interactions and as well as aid in development of strategies to control HLB.

Psyllid Yellows

"Psyllid yellows" was discovered in potato and tomato plants as a yellowing condition resulting from a "toxin" injected by the psyllid *Bactericera cockerelli* (BC) (Blood et al., 1933a; Richards, 1928, 1931) however, recently this disease was linked with *Candidatus* Liberibacter psyllaurous (CLps) infection, a phloem limited bacteria transmitted by *Bactericera cockerelli* (Hansen et al., 2008). *Bactericera cockerelli* is a hemipteren psyllid and is commonly known as tomato psyllid or potato psyllid. It can infest various solanaceous plant species such as tomato, potato and pepper (Hansen et al., 2008). Hansen (Hansen et al., 2008) showed that CLps could be transmitted into potato and tomato by different life stages of psyllid. CLps infected plants display symptoms such as leaf curling, yellowing or purpling of leaves, stunted growth, small size fruits, shortened internodes and sudden death (Cranshaw 1994; Pletsch 1947). The

CLps *16S rDNA* sequence is 97% similar to that of the HLB pathogen *Candidatus* Liberibacter asiaticus (Hansen et al., 2008). Comparison of genome sequences revealed 884 protein-coding sequences are common to CLas and CLso (or CLps) (Lin et al., 2011). Similarities in the pathogen transmission mechanisms and symptomology demonstrate the potential of psyllid yellows as a model disease system for HLB.

Development of control strategies by working directly with citrus species can be advantageous but it also poses several challenges such as A) long generation time that would extend the investigation and validation period of experimental results, B) lack of uniform distribution of HLB pathogen limits the effectiveness of pathogen detection and C) limited genomic resources will impede the study of candidate molecular interactions. Availability of a tractable and resource rich model plant species can not only overcome these challenges but may also deliver potential results in a rapid manner for further testing in the Citrus /HLB system. *Arabidopsis thaliana* has been used as a model plant species in various plant defense related studies (Davis and Hammerschmidt, 1993; Schlaich, 2011). In our study, we investigate the responses of *Arabidopsis* to *Candidatus* Liberibacter psyllaurous when transmitted by *Bactericera cockerelli* psyllids.

Arabidopsis **as a model plant system to study citrus diseases**

Arabidopsis thaliana is a small size, self-pollinating flowering plant belonging to family *Brassicaceae*. It is widely used in plant genetics, developmental and pathology research studies, mainly due to its desired features, such as small size, ease of

manipulation, efficient transformation, rapid life cycle, abundant seed production, sequenced and well-annotated genome, numerous mutant lines and, most importantly, extensive knowledge of its biology. Several studies have demonstrated the use of *Arabidopsis* as model plant species to understand molecular mechanisms of plant-pathogen interactions and develop disease resistant varieties (An and Mou, 2012; Lu et al., 2013; Zhang et al., 2010). In the study of citrus canker disease, *Arabidopsis AtNPR1* gene overexpression in Hamlin orange and Duncan grapefruit varieties resulted in ten-fold reduction of lesions formed by the pathogen *Xanthomonas citri subsp.citri* (Zhang et al., 2010). Using bioinformatics tools, the *Arabidopsis* NDR1 (NON-RACE SPECIFIC DISEASE RESISTANCE1) protein sequence was compared to the deduced citrus coding sequences to identify the ortholog of *NDR1* in citrus (*CsNDR1*) (Lu et al., 2013). Complementation of *Arabidopsis ndr1-1* mutant with the *CsNDR1* (a positive regulator of the salicylic acid (SA) pathway) induced resistance to a virulent *Pseudomonas syringae* strain and to the oomycete *Hyaloperonospora arabidopsidis* isolate Noco2 (Lu et al., 2013), demonstrating the potential of *Arabidopsis* resources to aid research on diseases of other plant species. Study of *Arabidopsis* and its interaction with *Xanthomonas citri subsp. citri*, a non-host pathosystem, revealed that genetic components of the SA pathway play a crucial role its non-host defense responses (An and Mou, 2012). Such studies demonstrate the potential of using *Arabidopsis* in understanding novel plant-pathogen interactions, which could further aid in development of disease resistant varieties in citrus.

SA, jasmonic acid (JA), and ethylene play vital roles in plant defense mechanisms. These three signaling molecules can work synergistically or antagonistically to induce or suppress defense-related responses. SA plays a critical signaling role in local resistance and systemic acquired resistance (SAR) against biotrophic pathogens and viruses (Vlot et al., 2009). The JA signaling pathway is triggered as a response to wounding, herbivores and necrotrophic pathogens (Kunkel and Brooks, 2002). Ethylene mediates defense signaling in response to herbivore feeding and pathogen depending on its life style (biotrophic/nectrophic) and the stage of infection process (Von Dahl and Baldwin, 2007; Van Loon et al., 2006). To understand the effect of these defense hormones on *Arabidopsis* phenotypic responses to CLps infection, we selected *npr1-1, pad4, coi-1, ein2-5* and transgenic *NahG*, lines compromised in SA, JA and ethylene defense signaling.

***NahG* (Salicylate hydroxylase)**

The salicylate hydroxylase *(NahG)* gene from the bacterium *Pseudomonas putida* encodes salicylate hydroxylase enzyme which converts salicylic acid into catechol (Yamamoto et al., 1965). Research studies on the role of SA in systemic acquired resistance (SAR) showed that *Arabidopsis thaliana* (Col-0) transformed with *NahG* gene has increased susceptibility to bacterial, fungal and other pathogens in comparison to wild type lines (Delaney et al., 1994; Gaffney et al., 1993). Such susceptibility was correlated to lower levels of SA and reduced expression of PATHOGENESIS RELATED-1

gene (*PR1*) (Delaney et al., 1994; Gaffney et al., 1993). Although deficiency in SA can predispose plants to increased susceptibility to pathogens or non-host pathogens, some reports also show that increased susceptibility in *NahG* transformed plants can result from catechol, a product resulting from SA degradation by salicylate hydroxylase (Van Wees and Glazebrook, 2003). In our study we used *NahG* transgenic line to determine if alteration in the SA pathway affects *Arabidopsis* response to *Candidatus* Liberibacter psyllaurous infection.

***NPR1* (*NON-EXPRESSOR OF PATHOGENESIS RELATED PROTEIN 1*):**

The *NPR1* gene plays an important role in the expression of *PR1* gene and consequently SAR in plants (Cao et al., 1994). The role of *NPR1* is quantitative and qualitative regulation of downstream genes in SA pathway such as *PR1* and *PR5* (Cao et al., 1994). Prior treatment of the *npr1-1* plants with SAR inducing SA, INA (2,6-dichloroisonicotinic acid) and inoculation with an avirulent strain of *Pseudomonas syringae* did not have any effect on its defenses and this line displayed severe disease symptoms to virulent *Pseudomonas syringae maculicola* ES4326 strain infection (Cao et al., 1994). Detailed molecular studies revealed nuclear localization of *NPR1* and its interactions with bZIP transcription factors that bind to the *as-1* (activation sequence-1) promoter element of *PR1* gene are vital in regulation of the SA signaling pathway (Kinkema et al., 2000; Zhang et al., 1999). Accumulation of NPR1 is essential for basal resistance whereas its degradation is necessary for systemic acquired resistance (Fu et

al., 2012). The NPR1 stability is regulated by NPR1 paralogues NPR3 and NPR4, and is dependent on cellular SA levels (Fu et al., 2012).

***PAD4* (*PHYTOALEXIN DEFICIENT 4*)**

Phytoalexins are low molecular weight secondary metabolites that act as antimicrobial compounds and are usually synthesized in response to pathogen elicitors (Paxton, 1981). Camalexin and rapalexin are the two known phytoalexins synthesized in *Arabidopsis thaliana* (Pedras and Adio, 2008). Phytoalexin synthesis is triggered upon the recognition of PAMPs (Millet et al., 2010) of necrotrophic as well as biotrophic pathogens (Ahuja et al., 2012; Thomma et al., 1999; Tsuji et al., 1992). The *pad4* mutant of *Arabidopsis thaliana* showed enhanced susceptibility to both virulent and avirulent strains of *Pseudomonas syringae* and had reduced levels of camalexin (Glazebrook et al., 1997). PAD4 functions as a regulator of signal transduction for camalexin biosynthesis (Glazebrook et al., 1997; Zhou et al., 1998). *PAD4* is a lipase like gene, which works in a signal amplification loop of SA pathway (Jirage et al., 1999) and its interaction with the *EDS1* gene is often required to trigger downstream defense responses (Feys et al., 2001). Study of *pad4* mutant line following CLps infection with respect to disease symptoms and bacterial titer, can provide novel insight into elicitor-triggered defense of *Arabidopsis* to CLps.

COI-1 (*CORONATINE INSENSITIVE-1*)

Coronatine is a chlorosis inducing toxin secreted by various strains of the bacteria *Pseudomonas syringae* (Mitchell, 1982; Mitchell and Young, 1978). It has a structure highly similar to jasmonates and can mimic MeJA (Methyl jasmonate) related effects such as senescence and root inhibition (Feys et al., 1994). JA and its derivatives play vital roles in processes such pollen development, fruit maturation, and root growth and defense signaling triggered by insects and pathogens (Creelman and Mullet, 1997; Turner et al., 2002). The *coi-1* mutant exhibits male sterility, fails to express JA-regulated genes effectively and resists JA-associated root growth inhibition (Feys et al., 1994). COI-1 is a F-box protein with 16 leucine rich repeats and is required for the JA signaling pathway (Xie, 1998). It is required for coronatine responses in *Arabidopsis*. Studies show that, in the presence of coronatine or jasmonoyl-isoleucine, *Arabidopsis* COI-1 interacts with Skp1-like 1, Skp1-like 2, cullin 1, and ring-box protein 1 (Xu et al., 2002; Yan et al., 2009) to form an E3 ubiquitin ligase known as the SCF^{COI1} complex which targets the JAZ protein (a repressor of JA signaling pathway) for degradation via the 26S proteasome pathway, thus activating JA signaling (Chini et al., 2007; Zhou et al., 2013). Various strains of *Pseudomonas syringae* synthesize coronatine as a virulence factor, which can trigger JA signaling and repress SA signaling, thereby effectively suppressing host defenses (Zhao et al., 2003).

EIN2-5 (ETHYLENE INSENSITIVE 2)

Ethylene is a gaseous plant growth regulator playing key roles in processes such as release of seed dormancy, flower and fruit ripening, leaf and fruit abscission, root growth and differentiation (Abeles et al., 1992). Many processes involving ethylene are concentration dependent and regulated by environmental and molecular cues (Pierik et al., 2006). Increased ethylene production is one of the early biochemical events in response to pathogen invasion or wounding that is required for triggering downstream defense (Boller et al., 1991), however in some cases ethylene also works as a pathogen virulence factor to overcome host responses by its excessive production which predisposes plant tissues to disease (Chagué et al., 2006; Hoffman et al., 1999). Ethylene is perceived by different membrane receptors specified by the ETHYLENE RESISTANT1 (ETR1), ETR2, ETHYLENE RESPONSE SENSOR1 (ERS1), ERS2 and ETHYLENE INSENSITIVE 4 (EIN4). These receptors further interact with a protein kinase, CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1), which in turn down regulates the ethylene signaling pathway (Chang et al., 1993; Gao et al., 2003; Hua et al., 1995, 1998). EIN2 is an important link that acts downstream of *CTR1* and is required to activate transcription factors EIN3/EIL1 or ERFs for triggering ethylene responses (Alonso et al., 1999; Roman et al., 1995). Studies show that *ein2* mutants are insensitive to endogenous and exogenous ethylene (Alonso et al., 1999; Roman et al., 1995) and that EIN2 is required for the activation of defense related genes such as basic chitinase, *PDF1.2* (Alonso et al., 1999).

In this study, I report a novel plant-insect-pathogen interaction of *Arabidopsis thaliana*, *Bactericera cockerelli* and *Candidatus* Liberibacter psyallurous. To study *Arabidopsis* response to the psyllid transmitted CLps pathogen, nineteen genetically diverse ecotypes were selected from the Clark's study (Clark et al., 2007). The selected ecotypes have few single nucleotide polymorphisms (SNPs) present in genes involved in basic biological processes but several SNPs in the defense related genes of which 60% of nucleotide-binding leucine-rich repeat (NB-LRR) and 15% of receptor like kinase (RLK) genes had larger effect producing SNPs (Clark et al., 2007). The objectives of this study were to determine if CLps can infect *Arabidopsis thaliana* ecotypes in addition to Col-0 and L*er*-1 and induce any disease related symptoms. Along with the wild type ecotypes, *NahG*, *npr1-1*, *pad4*, *coi-1*, *ein2-5* lines compromised in defenses were selected to determine if alteration in defense signaling can affect CLps transmission and infection in *Arabidopsis* and result in disease related symptoms.

METHODS AND MATERIALS:

Plant Material:

Seeds of 19 genetically diverse ecotypes listed in Table 1.3 were purchased from Arabidopsis Biological Resource Center (ABRC) and stratified for 72 hours at 4^{o}C in pesticide/fungicide free soil. All plants were grown under short day conditions (8 hours day at 23^{o}C /16 hours night at 22^{o}C) in an environmental growth chamber. At the 4-weeks stage, one plant per pot was infested with 10 nymphs (3rd-5th instar stage) and covered with organza bags (Fig 1.1A) until 3 weeks post infestation (wpi). Infested and non-infested plants were placed in separate bugdorms (BioQuip products, 1462W). An ecotype screening experiment (referred as "preliminary ecotype screen" in results section) was conducted with 7 replicates (5 infested and 2 non-infested) per ecotype to identify ecotypes likely to have higher or lower levels of resistance. To validate the preliminary ecotype screen experiment results, a repeat experiment (referred as "validation screen" in results section) was conducted with 18 replicates (15 infested and 3 non-infested) for each candidate ecotype. Leaves, stems, and inflorescence (siliques and flowers) were harvested at 3 wpi and 5 wpi time points to detect CLps infection. Seeds for all defense mutant / transgenic and wild type lines were provided by Dr. Thomas Eulgem (Department of Botany and Plant Sciences, University of California, Riverside). Except *coi-1*, all mutant lines were homozygous for the mutation. All *coi-1* plants were selected on 0.5X Murashige and Skoog (MS) medium supplemented with

45uM methyl jasmonate. At 2 weeks, plants of the same developmental stage were transplanted from MS media to soil. For the other mutant lines and wild type Col-0, seeds were sowed and cold stratified for 72-hours in pesticide/fungicide free soil. A total of 18 plants of each line were grown under short day conditions (8 hours day at 23^{o}C /16 hours night at 22^{o}C) in an environmental growth chamber. At the 4-weeks stage, 15 plants of each line were infested with 10 late instar stage nymphs and covered with mesh bags (Fig 1.1A). In all experiments, plants were continuously monitored to identify distinct phenotype changes. Tissue samples (leaves and stems) from all plants were collected at 3 wpi and between 5 wpi and 6 wpi.

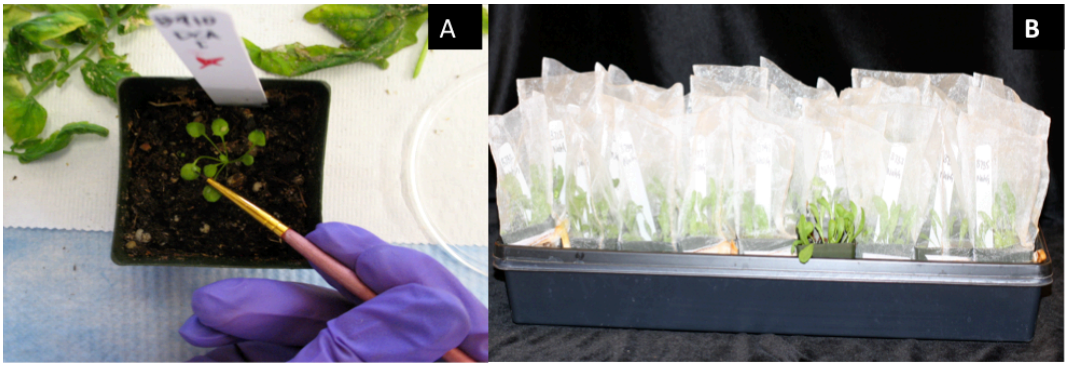

Figure 1.1: Infestation of *Arabidopsis* thaliana plants. A) Infestation of 4 weeks old plants with late instar stage nymphs using a small size brush. B) Plants covered with Organza bags post infestation.

Insect material:

A colony of *Bactericera cockerelli* (BC) was maintained on host *Solanum lycopersicum* var. Moneymaker plant (Source: Dr. John Trumble, Department of Entomology, University of California, Riverside) at 21-26^{o}C and 40-60% relative humidity

in environmental growth chamber. 90-98% of nymphs/adults were detected positive for CLps by real time quantitative PCR (qPCR) using *16S rDNA* primers (Table 1.1). In a small-scale experiment to evaluate different life stages of BC for CLps transmission efficiency, late instar stage nymphs and adult psyllid infected more CLps positive plants than younger nymphs.

Infestation of psyllids on *Arabidopsis* for *Candidatus* Liberibacter psyllaurous inoculation:

In all except one experiment (pilot experiment with only Col-0 and L*er*-1 ecotypes), a mixture of late instar nymphs was used for infestation. All psyllids used for infesting *Arabidopsis* plants were selected from symptomatic and CLps positive tomato plants. Each late instar stage nymph was selected based on its size and color. It was gently tugged to ensure that its stylet was free of the plant tissue, carefully picked with a paintbrush (Fig 1.1 A) and placed on an *Arabidopsis* leaf. Care was taken to avoid breakage of the psyllid stylet by choosing psyllids not feeding on leaves. After placing 10 nymphs, each plant was covered by a mesh bag (Organza bags) to restrict the psyllid movement to other plants. These plants were left covered until 2-3 weeks post infestation (Fig 1.1 B) to prevent psyllid escape.

DNA extraction:

The plant tissue samples were transferred into 2 ml tubes containing 2.5 mm zirconium beads (Research Products International Corp.) with 400 µl of extraction buffer (200 mM Tris (pH 7.5), 250 mM NaCl, 25 mM EDTA, 0.5% SDS). Samples were homogenized in MP Biomedicals FastPrep®-24 at setting of speed 6.5m/s and time 60 seconds and 400 µl of Chloroform: Isoamyl alcohol (24:1) was added to separate the aqueous phase. Isopropanol was used for DNA precipitation and the resulting DNA pellet was cleaned with 70% ethanol. Purified DNA was dissolved in 100 µl of sterile water and stored at 4°C. Samples with A260/A280 ratios between 1.7-2.00 were considered optimal for pathogen detection by qPCR.

Real time quantitative PCR (qPCR):

Two types of assays were used for qPCR A) SYBR green assay for testing ecotype responses to CLps infection and B) TaqMan assay for testing defense compromised lines responses to CLps infection. In the SYBR green assay, for each sample two separate reactions were set up with primer pairs for the CLps *16S rDNA* and *Arabidopsis ACT2* gene. All reactions were set up in 96 well plates (USA Scientific) and the reaction mixture (25 µl) contained IQ™ SYBR® Green Supermix (Bio-Rad#170-8880, proprietary reaction buffer, iTaq DNA Polymerase, SYBR® Green I dye), 0.25 µM of each of primers (Les33 and Les 34 or Les 37 and Les 38) (Table1.1) and 2µl of template DNA. Reactions were performed in Bio-Rad MyiQ™ or Bio-Rad iQ5™ thermo cyclers with cycling

conditions 95.0 °C for 10 minutes, followed by 40 cycles of 95.0 °C for 30 seconds and 58.0 °C for 55 seconds. Melting curves of PCR amplicons were generated with temperatures ranging from 55°C to 95°C with a 0.5°C increase in temperature every 10 seconds. In the TaqMan assay, all multiplex reactions were set up in 96 well plates (USA scientific) with each reaction mixture (25 µl) containing 1X reaction buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, pH 8.3, New England Biolabs), 0.25 mM dNTPs (Promega), 2.5 units of *Taq* DNA polymerase (New England Biolabs), 0.25 µM each of primers Les 33, Les 34, Les 37, Les 38, 0.15 µM each of Liberibacter and *ACT2* probes (Table 1.1), water added to bring the volume to 23 µl, and 2 µl of template. All reactions were performed in Bio-Rad iQ5 thermal cycler with cycling conditions 95.0 °C for 10 min, followed by 40 cycles of 95.0 °C for 30 seconds and 58.0 °C for 55 seconds. For both assays, each plate contained 2 to 4 non-template controls (NTC), and *Candidatus* Liberibacter psyllaurous-positive standard controls. All NTC controls were randomly placed to detect cross contamination among reactions. Comparison of SYBR green and TaqMan assays Cycle threshold (Ct) values generated for the same DNA samples showed no significant difference (Table S1.1), however evaluation of total cost per reaction indicated that the TaqMan assay was cost effective when testing more than 1000 samples for CLps. To confirm CLps positive plants, two criteria were used in all experiments: A) Ct values for multiple tissues of infested plant must be less than or equal to cutoff value of 33, B) when only one tissue of a plant showed a Ct value ≤ 33, the DNA sample was retested to confirm the value.

Table 1.1: Primers and probes used in real time quantitative PCR for *Candidatus* Liberibacter psyllaurous detection.

Gene ID*	Target genes	Target Organism	Primer IDs used in this study	Sequences (5' to 3')	Reference
EU812559	*16S rDNA*	CLps	Les33	TCGAGCGCTTATTTTTAA TAGG	(Hansen et al., 2008)
L22532	*16S rDNA*	CLas	Les34	TGCGTTATCCCGTAGAA AAAGGTAG	(Li et al., 2006)
821411	*ACTIN2*	*Arabidopsis*	Les37	GTTGGGATGAACCAGAA GGA	This study
821411	*ACTIN2*	*Arabidopsis*	Les38	GGCCTTTGGGTTAAGAG GAG	This study
L22532	*16S rDNA*	Liberibacter	Les39	FAM-AGACG GGTGA GTAACGCG-BHQ1	(Li et al., 2006)
821411	*ACTIN 2*	*Arabidopsis*	Les41 *ACT2* probe	Cy5-TTGCTCCTAAGAGC ACCTGTTCTT-BHQ3	This study

*NCBI gene ID or GenBank Accession ID number

Standard Curve for CLps *16S rDNA* and *Arabidopsis ACT2* gene

A *Candidatus* Liberibacter psyllaurous standard curve was generated using Ct values of 10-fold serial dilutions of plasmid DNA containing CLps *16S rDNA* (EU812559) and the respective log of copy numbers of target DNA. Standard curve of *Arabidopsis ACT2* gene was generated using a 10 fold serial dilution of a plant sample (# GT711) with DNA concentration (330ng/µl) and the respective log of copy numbers of target DNA. Copy number calculations for both genes are described in supplementary methods (TableS1.2-Table S1.4). All standard DNA samples and dilutions were done in three technical replicates for qPCR to validate the reliability of Ct values. Each qPCR plate contained serial dilutions of the *16S rDNA* standard DNA to determine the efficiency of the qPCR run. The range of efficiencies was 90-102% for all qPCRs detecting CLps positive / negative samples.

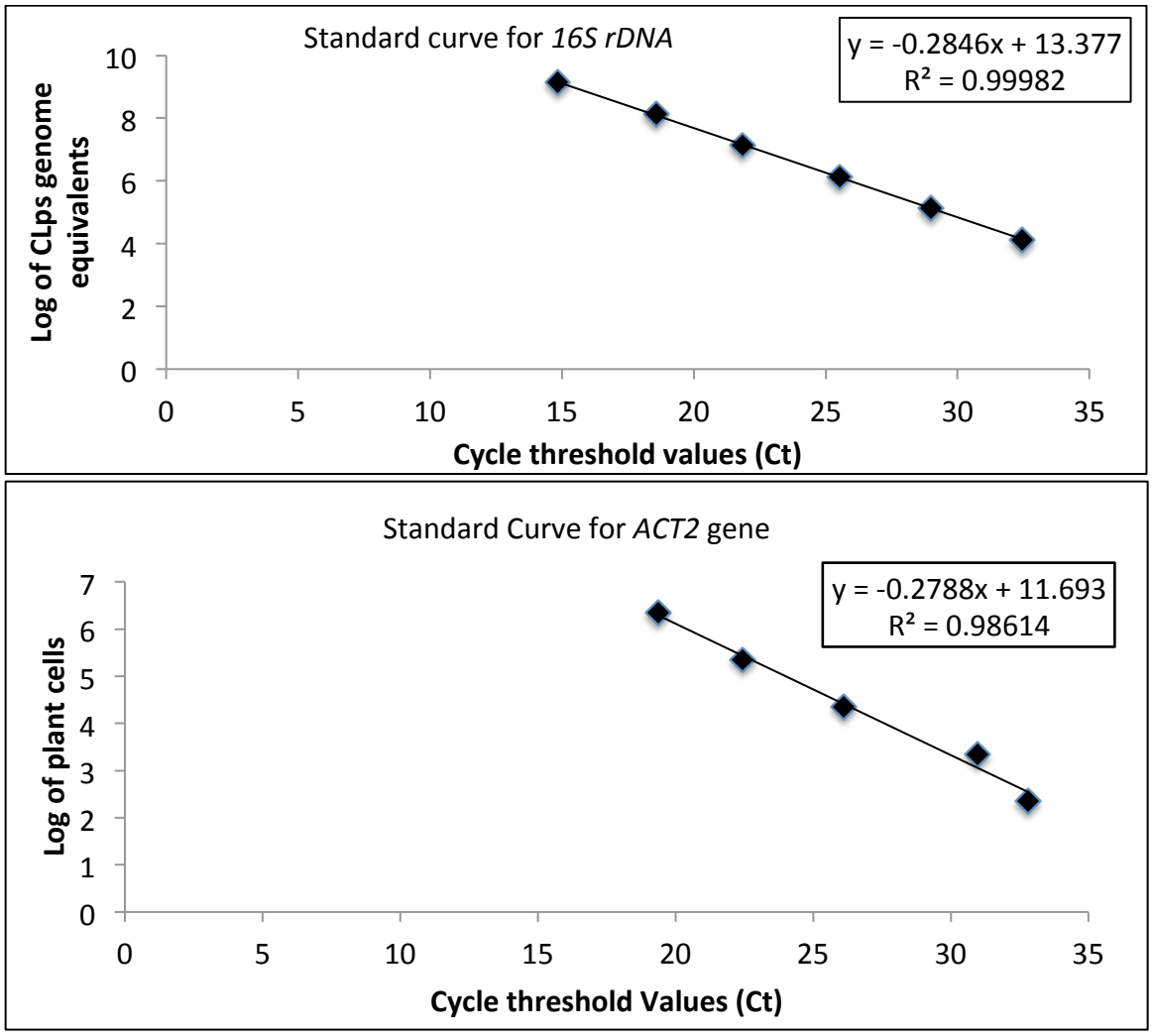

Figure 1.2: Standard curve of *Candidatus* Liberibacter psyllaurous *16S rDNA* and *Arabidopsis ACTIN2* gene

RESULTS:

***Bactericera cockerelli* transmits *Candidatus* Liberibacter psyllaurous into *Arabidopsis thaliana*, but fails to survive and reproduce.**

Different *Solanaceous* plants such as tomato, pepper and potato serve as successful hosts to BC and its endosymbiont CLps. In an attempt to determine if BC can survive and transmit CLps into *Arabidopsis thaliana*, 3 replicates of Col-0 and 3 replicates of L*er*-1 plants were infested with a mix of early instar stage nymphs (1^{st} and 2^{nd} instar stage), mix of late instar stage (3^{rd} to 5^{th} instar stage) nymphs and adults. All plants were visually assessed for psyllid survival, however BC psyllids (adults and nymphs) failed to survive on *Arabidopsis thaliana* plants. No BC psyllids at nymphal stages (early and late instar stages) survived more than a week and very few adult psyllids survived until 2 wpi. To determine if BC infestation results in the transmission of CLps into *Arabidopsis*, different plant tissues such as leaves, stems and inflorescence (flowers and siliques) were tested for CLps infection using CLps specific primers. Leaves, stems and inflorescences were found to be positive at 3 wpi (Ct values ≤ 33). For both the Col-0 and L*er-1* ecotypes, stems had significantly higher bacterial cell numbers compared to leaves (Student t-test, $p<0.05$, Table 1.2) when infested with late stage nymphs and adults of BC psyllid.

Table 1.2: Average number of *Candidatus* Liberibacter psyllaurous genome equivalents per gram detected in leaves and stems of Col-0 and L*er*-1 at 3 weeks post infestation by nymphs and adults in preliminary experiment.

Ecotypes	Leaves	Stems
L*er*-1*	1.18×10^7	1.10×10^{10}
Col-0*	6.10×10^7	2.74×10^9

*Significant difference ($p<0.05$, student t-test) in CLps titer in the leaves and stems for both ecotypes.

In all experiments, *Bactericera cockerelli* psyllids failed to survive on *Arabidopsis*, however the detection of CLps in plants indicated its' feeding on the plants. Apparent feeding behavior of psyllids was also noted on plants. Detection of CLps in stem (not developed at infestation) in higher cell numbers indicates movement of bacteria from leaves (point of inoculation) into new growth (inflorescence stems). This was the first evidence showing that CLps can multiply in *Arabidopsis*. However, not all segments of a stem were detected positive for CLps, indicating non-uniform distribution of pathogen. Interestingly in this experiment, at 3 wpi no distinct symptoms were observed among the CLps positive plants. In addition to PCR amplification of target CLps DNA, sequencing was performed with the amplified DNA and presence of CLps was confirmed in *Arabidopsis* plants.

Responses of genetically diverse ecotypes to *Candidatus* Liberibacter psyllaurous infection were inconsistent.

To determine if CLps can infect other ecotypes in addition to Col-0 and L*er*-1, nineteen genetically diverse ecotypes were selected from a collection of ecotypes studied for genome variation and effects of sequence polymorphisms in evolution (Clark

et al., 2007). These accessions were screened to determine the responses to CLps infection via psyllid transmission. Results indicate variability in the percentage of infected plants and bacterial titers. All the selected ecotypes were infected with CLps at 5 wpi and had variable bacterial genome equivalents in different tissues in ecotype screening experiment (Table 1.3). Statistical analysis indicated no significance differences in the percentage of infected plants of ecotypes (Chi-Square test, $p>0.05$). The CLps genome equivalents were determined using a standard curve method for all plant tissues and ecotypes. The average bacterial genomes per plant cell ranged from 0.01 to 1685. The statistical analysis was performed with the log values of average bacterial genomes/per plant cells for all ecotypes. Although, the analysis indicates significant difference between bacterial titers of plants among ecotypes (one way ANOVA, $p<0.05$), these results must be interpreted with caution. The number of replicates was too small to determine resistance or susceptibility of ecotype and factors such as lack of uniform distribution of pathogen in plants and variation in developmental stage of tissues among ecotypes can influence detection of CLps bacteria. Even with high bacterial titer, no CLps infected plant showed any distinct symptoms at 5 weeks post infestation. To validate the ecotype preliminary screen results, nine ecotypes were selected based on highest or lowest number of infected plants or high bacterial titer or low bacterial titer. Table 1.4 shows the comparison of results from preliminary and validation ecotype screens wherein the inconsistency in ecotype responses was evident.

This inconsistency can be attributed to the variability in CLps transmission behavior among nymphs possibly resulting from *Arabidopsis* non-host resistance. In addition to resistance to psyllids, the possibility that genetic variation can influence the CLps infection must not be discarded.

Table 1.3: Responses of genetically diverse ecotypes to CLps infection.

Accession #	Geographic locations	Ecotypes	Percentage of infected plants	Minimum and maximum CLps genome equivalents / plant cell detected in any tissue
CS22683	Estonia	Est-1	100	0.04 – 3.81
CS22684	Portugal	Fei-0	100	1.32 – 10
CS22695	Sweden	Lov5	100	0.11 – 3.36
CS22686	Poland	Ler-1	100	0.1 – 34
CS22694	USA	Van-0	80	0.02 – 0.03
CS22679	Ireland	Bur-0	60	6.7 – 8.73
CS22687	England	Nfa-8	60	2.67 – 20
CS22688	USA	Rrs-7	60	0.4 – 5.49
CS22678	Czech	Br-0	40	16 – 26
CS22682	Cape Verde	Cvi-0	40	0.14 – 3.83
CS22680	Portugal	C24	40	8.3 – 17
CS22685	Germany	Got7	40	0.4 – 6.3
CS22689	USA	Rrs-10	40	0.04 – 17
CS22690	Tajikistan	Sha-1	40	0.08 – 0.1
CS22691	Finland	Tamm-2	40	0.01 – 0.08
CS22693	Japan	Tsu-1	40	0.05 – 24
CS22677	Czech	Bor-4	40	13 – 1685
CS22681	Germany	Col0	20	0.12
CS22692	Spain	Ts-1	20	0.3

Note: All plants (5 infested and 2 non-infested controls) were tested for CLps infection using CLps 16S rDNA primers. QPCR Cycle threshold (Ct) cut off value of 33 was used to identify CLps positive plants and to determine average bacterial genome equivalents/plant cell.

Table 1.4: Comparative analysis of responses of genetically diverse ecotypes to CLps infection in ecotype preliminary screen and validation screens.

Ecotypes	Ecotype preliminary screen results at 5 wpi		Validation screen results at 5 wpi	
	Percentage of CLps-Infected plants	Genome equivalents/ plant cell in any tissue*	Percentage of CLps-Infected plants	Genome equivalents/ plant cell in any tissue*
Col-0	20	0.1	33	4.5 – 3766
Van-0	40	0.02 – 0.03	66	0.02 – 330
Tamm-2	40	0.01 – 0.08	7	17.8
Rrs-7	60	0.4 – 5.49	0	0.0
Nfa-8	60	2.67 – 20	13	15 – 134
Lov-5	60	0.11 – 3.36	46	0.16 –301
Est-1	100	0.04 – 3.81	33	0.03–1.74
Ler-1	100	0.1 – 34	0	0.0
Fei-0	100	1.32 -10	0	0.0

* Minimum and maximum of CLps genomes detected in a qPCR reaction. QPCR Cycle threshold (Ct) cut off value of 33 was used to identify CLps positive plants and to determine average bacterial genome equivalents/plant

***Candidatus* Liberibacter psyllaurous infects wild type Col-0, L*er*-1 and defense compromised lines but no significant difference was detected in the CLps-infection related responses between the defense compromised and wild type lines**

An intricate and systematic network of defense pathways plays a crucial role in the plant immune system**.** These pathways often work synergistically and sometimes antagonistically to trigger plant defenses. Salicylic acid, jasmonic acid and ethylene are the three key signaling biomolecules responsible for inducing defenses against various types of plant pathogens. To understand the importance of these signaling pathways in CLps-triggered plant responses and identify a line showing distinct CLps related symptoms; lines compromised in defense signaling of the SA, JA and ET pathways were examined for the changes in phenotype and bacterial titer. The *NahG*, *ein2-5*, and wild type Col-0 lines had 53-60% (Fig 1.3A) infected plants and showed a significant increase in bacterial titer at 6 wpi (Fig 1.3C-D, Student's *t*-test, $p<0.05$). A considerable increase in bacterial genomes from 3 wpi to 6 wpi was also evident (Fig 1.3C-D) in other defense-compromised lines. Wild type Col-0 had higher bacterial titer (CLps genome equivalents) compared to defense compromised lines and similar number of infected plants as *NahG* (SA defense compromised) and *ein2-5* (ethylene mutant). The high titer in Col-0 can be attributed to the type of tissue-detected positive for CLps, which were mostly stems. The CLps bacteria inhabits phloem tissues of plants so it is very likely that it would be present in high numbers in the stem tissue than leaf. Six of eight CLps positive plants

and seven of nine CLps positive plants tested positive based on stem tissue from lines Col-0 and *NahG* respectively (Fig 1.3B). With *ein2-5* line, 7 of 8 CLps-infected plants were detected positive for CLps infection based on only leaf tissues testing. It was interesting to observe that in case of *coi-1* line, with an exception of only one plant, all tissues of all CLps infected plants were detected positive and had high bacterial titers. Overall analysis of bacterial genomes / plant cell for all lines indicates that within an individual line, plants can have as low as 0.06 bacterial genomes / plant cell and high as 259 bacterial genomes/plant cell. (Supplementary table S1.5 shows the average, median and standard deviation of bacterial genomes/plant cell). No significant difference was detected in the log transformed bacterial genomes/plant cell and bacterial genomes / gram of tissue among the lines ($p>0.05$, one way ANOVA). Individual comparative analysis of defense-compromised lines with the wild type Col-0 line shows no significant difference in number of bacterial genome/plant cell (Student's t-test, $p>0.05$).

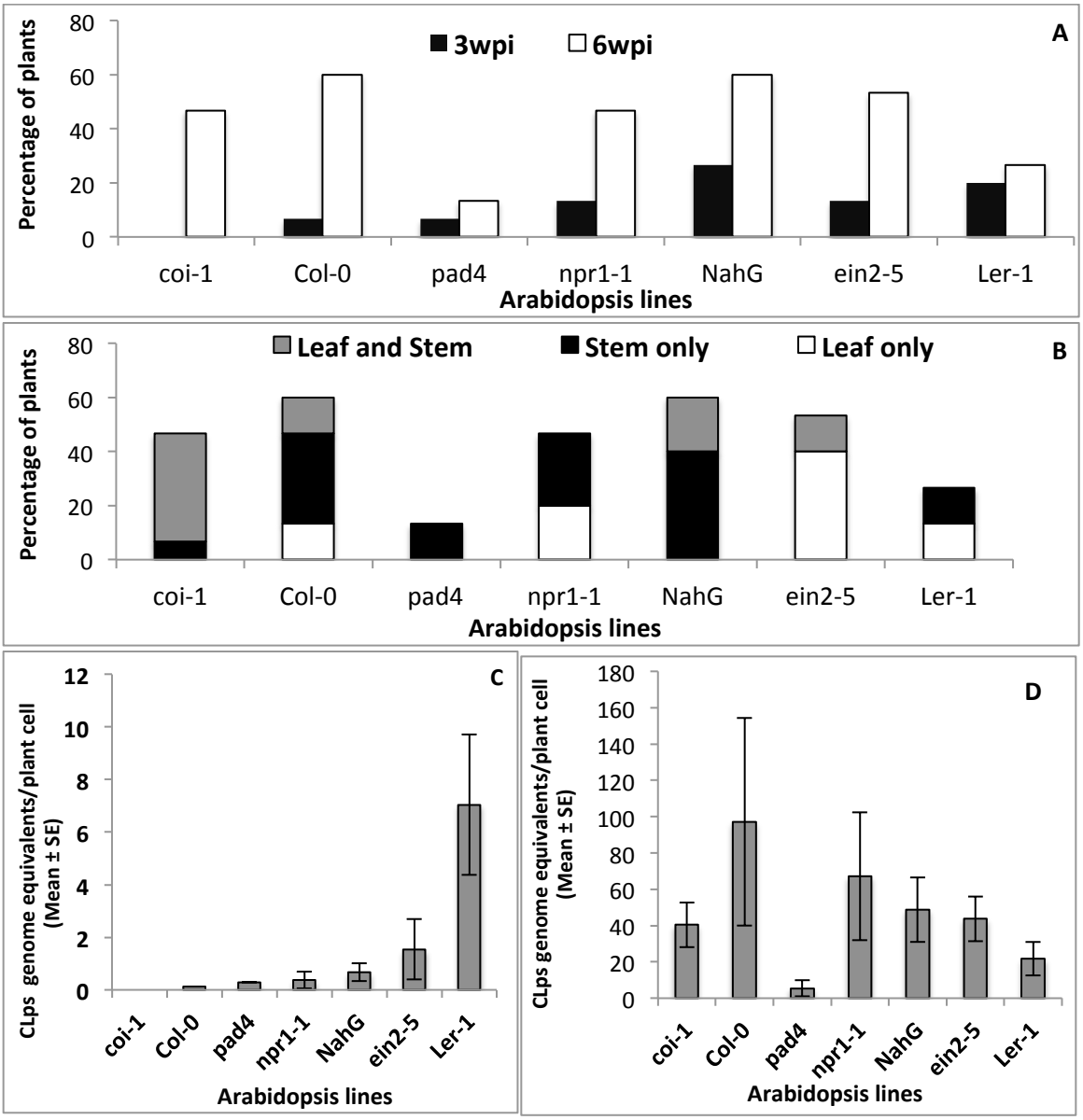

Figure 1.3 A-D: Responses of defense compromised and wild type *Arabidopsis* lines to *Candidatus* Liberibacter psyllaurous infection. A) Percentage of CLps infected plants detected by any tissue of infested plants at 3wpi and 6wpi, B) Percentage of psyllid infested plants detected as CLps-infected by stems, leaves and both tissues at 6wpi, C) CLps genome equivalents /plant cell estimated at 3wpi for different lines (Data represent mean ± standard errors for all CLps-positive plants, $p>0.05$, one way ANOVA) D) CLps genome equivalents / plant cell with standard error bars estimated at 6wpi for different lines (Data represent mean ± standard errors for all CLps-positive plants , $p>0.05$, one way ANOVA). ***NOTE:*** *The coi-1 mutant line was studied in a separate experiment along with wild type Col-0* and no significant difference in CLps genomes/plant cell was detected at 6wpi between two lines. *In this experiment unlike coi-1, plants of Col-0 line showed considerable variation in CLps genomes/plant cell. For these Col-0 plants, the range of CLps genomes/ plant cell detected was 0.44 -2009, with median of 66.83 CLps genomes/plant cell.*

***Candidatus* Liberibacter psyllaurous infection results in distinct and novel symptoms in *Arabidopsis* defense mutants and wild type Col-0 starting at 5-6 weeks post infestation**

The pilot experiments with Col-0 and L*er*-1 showed that *Candidatus* Liberibacter psyllaurous infects *Arabidopsis thaliana* via psyllid transmission and can be detected as early as 2 wpi. In the preliminary and validation ecotype screening experiments, the percentage of CLps-positive plants was variable among ecotypes, however, no CLps positive plants showed any distinct symptoms at 5 wpi. In an effort to understand if any alteration in plant defenses can trigger symptoms, observation of defense mutant lines was extended to 6 wpi-7 wpi. Interestingly, CLps positive plants of all mutant lines *NahG*, *pad4*, *ein2-5, npr1-1, coi-1* and wild type Col-0 exhibited at least one distinct symptom such as purple coloration, curled central axillary leaves and excessive growth of small axillary leaves and clusters of cauline leaves at 6 wpi- 7 wpi (Fig 1.4). In general, discoloration along the veins or lower surface of leaf was distinctly visible by 6 wpi in infected plants (Fig 1.4E). Few plants of *coi-1* line also showed some discoloration on stems. Leaf curling was one of the first symptoms visible on CLps-infected plants (Fig 1.4A and1.4B). Young/new leaves tend to curl downwards on infected plants. Another notable symptom was the overgrowth of small size cauline and axillary leaves (Fig 1.4D) distinctly evident on CLps positive plants of *NahG, npr1-1* and *pad4* mutant lines.

Most of the symptoms appeared on infected plants after 5 wpi even when they were detected positive at 3 wpi indicating latency in symptom development. Table 1.5 summarizes the frequency of symptoms observed in the defense compromised and wild type lines.

Table 1.5: Percentage of plants showing symptoms in defense compromised and wild type lines at 6 wpi.

	Number of plants*		**Discoloration (%)**		**Axillary/Cauline leaf Overgrowth (%)**		**Leaf Curling (%)**	
	A	**B**	**A**	**B**	**A**	**B**	**A**	**B**
Col-0	6	9	33	100	0	11	0	11
Ler-1	11	4	0	75	0	25	18	75
NahG	6	9	0	100	0	44	33	89
ein2-5	7	8	43	100	0	38	0	100
npr-1	8	7	0	43	0	14	0	57
pad4	13	2	0	100	0	100	0	100
coi-1¥	8	7	57	63	0	38	0	38
Col-0¥	10	5	0	60	0	0	20	20

*Total number of plants in A and B treatments A = Plants infested with psyllids and negative for CLps infection, B= Plants infested with psyllids and positive for CLps infection, ¥- Separate experiment.

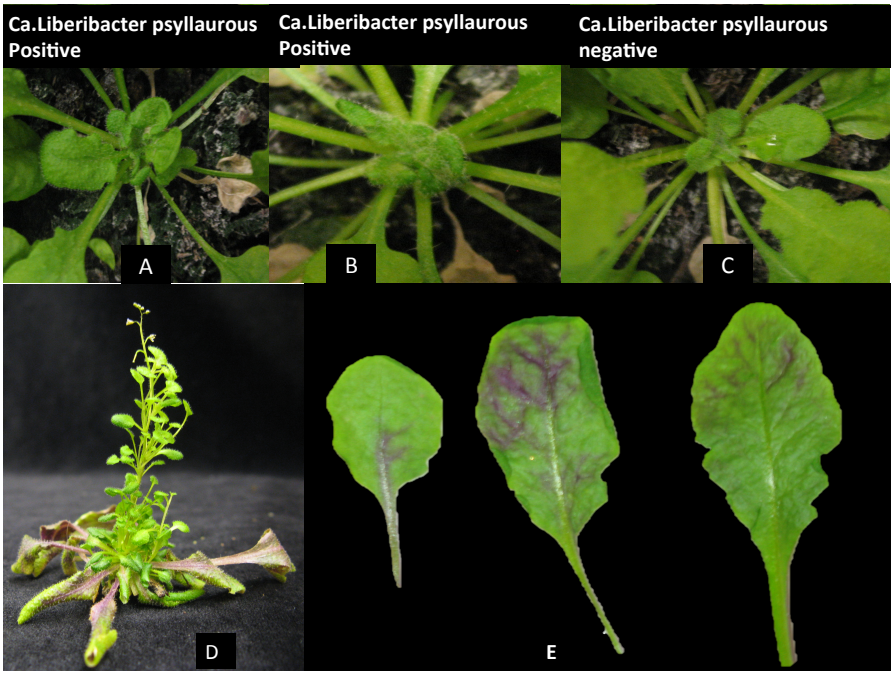

Figure 1.4: *Arabidopsis* symptoms in response to CLps infection.
A-B) Curling of young leaves and slow growth of central axillary leaves in CLps-infected plants C) No curling or slow growth observed in *Arabidopsis* plants detected negative for CLps D) Slow growth of stems and excess growth of small axillary and cauline evident on stems and on rosette structure of infected plant. E) Asymmetric discoloration or purpling of leaves observed on infected plants.

Comparative analysis of cycle threshold values and the corresponding number of Liberibacter genomes / gram of fresh tissue in *Arabidopsis and Citrus* indicate similarity in the number genomes of Liberibacter species.

The Ct values of different tissues and corresponding variable numbers of CLps genomes provide a strong evidence of non-uniform distribution of CLps in *Arabidopsis*. In this study, we have used the ratio of bacterial genomes to plant cell to determine the differences in plant immunity to CLps. Although, the ratio varies considerably when multiple plants of single ecotypes or lines were compared, it provides a reliable estimate

of pathogen spread when different tissue types are analyzed simultaneously. Another approach to quantification is measuring bacterial genomes per gram in plants. To determine if Ct values and corresponding bacterial CLps genomes in *Arabidopsis* is comparable to Citrus/HLB system, I compared the Ct values generated in this study with the Ct values published in HLB related literature (Li et al., 2009) (Table 1.6). For almost all cycle threshold values, the number of CLas genomes per gram fresh tissue of citrus was twice than the number of CLps genomes per gram fresh tissue of *Arabidopsis*. This comparison shows a considerable similarity between CLas/Citrus and CLps/Arabidopsis pathosystems. Another aspect to be considered with such comparison is the time point of Liberibacter detection. We can reliably detect CLps with Ct values as low as 17 by 6 wpi in *Arabidopsis*, which indicates the rapid spread or mutliplication of Liberibacter in *Arabidopsis.*

Table 1.6: Comparison of Liberibacter genomes per gram tissue of Citrus with *Arabidopsis*

Cycle threshold values	**Liberibacter genome equivalents /gram of tissue**	
	Citrus/HLB [a]	*Arabidopsis*/CLps
18.38	1.78E+11	7.00E+10
19.52	8.41E+10	3.32E+10
24.41	3.33E+09	1.35E+09
26.8	6.89E+08	2.81E+08
28.89	1.75E+08	7.14E+07
30.56	6.00E+07	2.39E+07
31.45	3.20E+07	1.33E+07

a- Ct values and corresponding genomes/gram for Citrus/HLB (Li et al., 2009).

DISCUSSION:

We used *Bactericera cockerelli* and CLps as a model vector-pathogen system to determine their potential in development of high throughput chemical screening for identifying defense-inducing compounds. With well-established protocols and extensive genomic resources *Arabidopsis thaliana* has been successfully used in studies involving high throughput chemical screening to study biological processes or identify defense-inducing compounds. In this study, various lines of *Arabidopsis* were investigated for their response to CLps infection and to determine their potential in high throughput chemical screening. Through this study, we report the first evidence of transmission of CLps in *Arabidopsis thaliana* by psyllid and CLps-related symptoms. Our preliminary study showed successful CLps transmission into *Arabidopsis* lines Col-0 and L*er-1* by adult psyllid *Bactericera cockerelli*. Different stages of *Bactericera cockerelli* psyllid were evaluated to determine the optimum CLps transmitting stage for *Arabidopsis* in order to maximize the number of CLps positive *Arabidopsis* plants. Late instar nymphs and adult psyllids transmitted CLps to a higher percentage of plants than early instar stage nymphs. Our observations indicate that nymphs do not survive on *Arabidopsis* for more than 2 weeks. The tendency of psyllids to move away from the plant indicates incompatible interaction between *Bactericera cockerelli* and *Arabidopsis.* Such incompatible interactions usually result from preformed and basal defenses. Preformed defenses are usually in the form of physical and chemical barriers at the leaf surface that play an important role in deterring herbivore feeding and colonization. Often glandular

trichomes secrete volatile and non volatile secondary metabolites to deter herbivore feeding and growth (Wagner et al., 2004). It could be interesting to understand the genetic basis of such defenses that deter psyllid feeding to use as models for control measures against Asian citrus psyllids.

Our investigation to determine if genetic variation among different *Arabidopsis* accessions affects their susceptibility/ tolerance/resistance to CLps was inconclusive due to high variation within and between experiments. All plants were tested before and after infestation and CLps positives were detected only post infestation indicating psyllid feeding and CLps transmission into plants. In the preliminary ecotype screen, some plants of all ecotypes tested positive for CLps infection and bacterial populations were quite variable. In the validation screen, ecotypes Fei-0 and L*er*-1 had no plant detected as positive for CLps in contrast to the preliminary results of 100% infected plants for both ecotypes. Such inconsistency was observed for all the selected ecotypes; therefore we could not confirm effects of genetic variation on defense responses. However, all ecotypes had some CLps positive plants in all experiments. At 5 wpi (plant age was 9 weeks) when various tissues were harvested from all plants, we were unable to identify any distinct disease related symptoms such as leaf discoloration observed in Liberibacter infected tomato plants.

To determine if *Arabidopsis* is tolerant to CLps, an experiment with defense compromised lines and wild type lines Col-0 and Ler-1 was conducted with an extended time line of six to seven weeks post infestation. Salicylic acid, jasmonic acid, ethylene and other phytohormones play crucial roles in mediating plant defense responses when exposed to different pathogens. In case of HLB-citrus, various genes involved in SA, JA and ET regulated defense pathways were significantly differentially expressed as the result of CLas infection (Albrecht and Bowman, 2008, 2012). Studies suggest that in this compatible interaction of citrus-CLas, plants develop symptoms due to the plant's inability to induce a sufficient magnitude of defenses (Albrecht and Bowman, 2012). The role of salicylic acid as an endogenous signaling hormone that activates local and systemic resistance to bacterial infection is well documented (Vlot et al., 2009). In our study we selected two SA deficient lines *NahG* and *npr1-1* to determine its role of SA in response to CLps infection. Studies link the lower SA levels of *NahG* line (SA deficient) and resulting susceptible phenotype to the role of SA in activating defenses in response to bacterial infection (Delaney et al., 1994; Van Wees and Glazebrook, 2003). At 6 wpi, 9 of 15 psyllid infested *NahG* plants were detected positive for CLps with an average of 48 CLps genome equivalents per plant cell and 7 of 15 infested *npr1-1* plants positive for CLps with an average of 67 CLps genome equivalents per plant cell, Both *NahG* and *npr1-1* lines displayed distinct symptoms including leaf discoloration on veins (asymmetric purple coloration), leafy overgrowth in center, or clusters of small cauline leaves. Although, both lines were compromised in SA defense pathway, their

susceptibility to CLps infection (bacterial genomes/plant cell, symptomology) was similar to wild type Col-0 line at 6 wpi. These results emphasize the complexity of plant defense against Liberibacter and potential requirement of other regulatory defense networks. Ethylene and jasmonic acid mediated defense pathways often work synergistically and trigger defenses in responses to a range of pathogens such as fungi, bacteria and herbivores. We tested two mutant lines *ein2-5* (ethylene defense mutant) and *coi-1* (JA defense mutant) for responses to CLps infection. At 6 wpi, 8 of 15 infested *ein2-5* plants and 7 of 15 infested *coi-1* plants were detected positive for CLps infection and had 43 CLps genome equivalents per plant cell and 40 CLps genome equivalents per plant cell respectively. With *ein2-5* line, all plants were detected positive by leaf tissues and all infected plants showed severe discoloration compared to other lines at 6 wpi. In the *coi-1* line, no plant was detected positive at 3 wpi but by 6 wpi 46.67% of infested plants tested positive for CLps. An interesting aspect of the *coi-1* plants was that all CLps positive plants had multiple tissues tested positive for CLps with high bacteria titers irrespective of tissue type. This was consistently observed across all CLps positive coi-1 plants indicating a possibility that these plants were fed by psyllids with better efficiency. Coi-1 is a receptor of JA and plays a crucial role in JA related functions such as plant defenses to insects or other herbivores. Studies show that *coi-1* mutant line has increased susceptibility to insects and other pests (Abe et al., 2009, 2013), so it is possible that mutation in *Coi-1* could have altered *Arabidopsis* response to psyllid feeding efficiency and consequently transmission of CLps. It was evident that bacterial

titer increased over time in all CLps positive plants irrespective of genetic background. Within the same genetic background, as the pathogen titer increased the severity of symptoms also increased. However, it was intriguing that the symptom severity did not correlate with bacteria titer when *coi-1* infected plants (Fig 1.5A) were compared to *ein2-5* infected plants (Fig1.5B). All the tissues harvested from the *coi-1* plant were positive for CLps and both plants had high bacteria (41 and 43 CLps genomes/plant cell), but *ein2-5* displayed more severe symptoms. Based on a similar observation for multiple plants of both lines, it is evident that there is no correspondence in intensity of symptoms and bacterial titer of plants of same species. Also, this result indicates a possibility that ethylene pathway may play an important role in *Arabidopsis* response to CLps infection. Out of all mutant lines, *pad4* had the lowest number of infected plants (2 of 15 infested plants) and only one plant displayed strong symptoms. In this study, we have used single mutant lines compromised in defenses. It could be interesting to check if any alteration or manipulation of multiple genes (double or triple mutant) involved in defense pathways especially SA or ET can affect *Arabidopsis* response to CLps infection.

Figure 1.5: Relation between symptoms and bacterial titer in two different defense mutant lines. A) CLps positive *coi-1* mutant plant with 41 CLps genomes/plant cell and B) CLps positive *ein2-5* mutant plant with 43 CLps genomes/ plant cell.

In all lines, excessive growth of small cauline or axillary leaves occurred only on CLps infected plants. Purple coloration near veins and central leaf curling were typically the first symptoms observed on infected plants. All CLps positive plants displayed symptoms at 10 weeks stage when they are closer to senescence. There is a likelihood that increased severity of symptoms results from increased bacterial titer or more rapid senescence or both. Most solanaceous plant species such as *Solanum lycopersicum* (tomato), *Solanum tuberosum* (potato), and *Capsicum annuum* (pepper) are affected by psyllid yellows. In tomato, CLps infection results in severe discoloration or yellowing of leaves, leaf curling, no fruits or overproduction of small sized fruits, and overall significant deterioration of plant health leading to plant death. Similarities in symptomology of *Arabidopsis* infected with CLps and tomato infected with CLps were remarkable. Discoloration, slow growth and small size young leaves were distinctly

evident on *Arabidopsis* and tomato infected with CLps. In addition to resemblances in phenotypic changes with PY, the latency in symptom development in CLps-infected *Arabidopsis* is highly similar to CLps-infected tomato and HLB infected citrus. The comparative analysis bacterial titer revealed that *Arabidopsis* has almost similar number Liberibacter (CLps) genomes when compared to Liberibacter genomes in citrus for same Ct values. This is a remarkable similarity in both pathosystems. Most of the CLps-infected *Arabidopsis* even with high bacterial titer did not display distinct symptoms until 6 wpi, but became distinct by 7 wpi. This delay in symptom development could possibly result from lack of uniform bacterial infection in plants and or plant's responses to CLps infection.

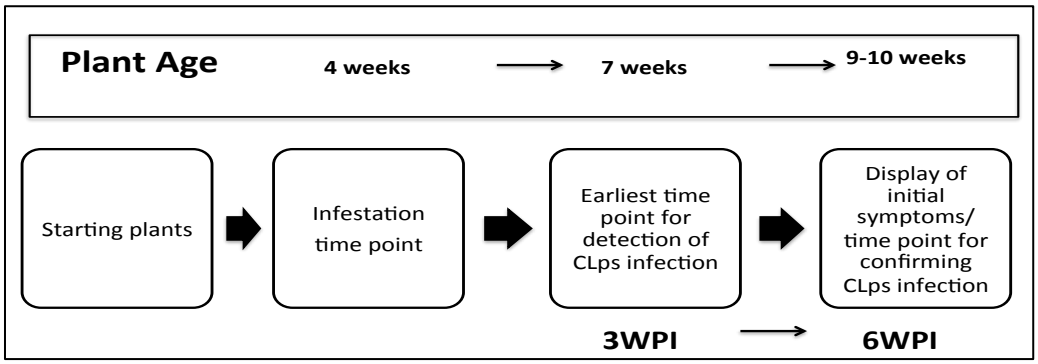

Figure 1.6: Timeline for detection of CLps in *Arabidopsis thaliana*

In this study we determined that CLps can infect *Arabidopsis* and based on similarities in symptomology and bacterial infection with Citrus /HLB, it has a potential to be used in assays for understanding interactions between plants and *Candidatus* Liberibacter. The total time line for an *Arabidopsi*s and CLps/ BC assay is 9 to 10 weeks (Fig 1.6), which is

comparatively shorter than most reports on experimental assays of the citrus/HLB system (Albrecht and Bowman, 2008, 2012; Kim et al., 2009). However, the inconsistent responses for ecotypes demonstrate that CLps transmission is dependent on nymph feeding efficiency. Either the frequency of feeding and transmission is so low that infestation with 10 nymphs per plant results in many plants not becoming infected, or the physiological state of the replicate plants within a line makes some more resistant than others. Our inability to consistently infect a high percentage of plants is a major obstacle to analysis of the response of *Arabidopsis* to CLps. Possibly infestation with more than 10 nymphs per plant would increase the percentage infected plants, but manual application of nymphs is slow so applying more nymphs would reduce the number of plants that can be tested. To overcome the challenge of incompatible interaction and maximize the possibility of CLps transmission into *Arabidopsis*, it could be interesting to determine if exposing plants to large numbers of adult psyllids can result in consistent numbers of CLps positive plants. For example, placing a tray of plants in a chamber containing more than 1000 psyllids for a week.

All the observations indicate that *Arabidopsis* is susceptible to CLps infection when psyllids were forced to feed on them. The increase of bacteria in plants over time indicates that CLps can multiply in *Arabidopsis*. However, due to non-host resistance to the psyllid vector these plants may not be exposed to Liberibacter in nature. In nature, typically plants respond to various potential pathogens and pests at different defense

levels that work in a highly coordinated manner. Preformed defense barriers such as waxy cuticle, secondary metabolites, and peptides act as first level of defense and work as deterrents to provide non-host resistance to the potential pathogens. If a pathogen successfully overcomes the preformed defenses, plants may recognize these pathogens by its elicitors also known as pathogen associated molecular patterns (PAMP) using pattern recognition receptors (PRRs) (Jones and Dangl, 2006). Such recognition triggers downstream mitogen activated kinase activity and elicits resistance to pathogens. However, occasionally in the course of evolution, pathogens can overcome PAMP triggered immunity by using virulence factors also known as effectors (Jones and Dangl, 2006). To counteract such effector-based susceptibility, plants can evolve disease resistance genes (R genes), which can interact with these effectors and trigger strong downstream defense against these pathogens (Jones and Dangl, 2006). In case of CLps infection, defense response elicited in plants may be basal or PAMP resistance, and R-gene specific resistance may be lacking due to lack of co-evolution. In further studies (Chapter 2), we examine response of *Arabidopsis* to CLps infection to gain insights into its defense mechanism.

CONCLUSION:

Despite low transmission rate, *Bactericera cockerelli* transmits *Candidatus* Liberibacter psyllaurous into *Arabidopsis thaliana*. Irrespective of genetic background, bacterial titer increases over time in plants and bacteria can be detected in new growth of plants. We used five defense compromised (single mutant) lines to understand *Arabidopsis* defense response to CLps, however it could be interesting to include more mutant (single or double) lines to dissect the intricate responses to CLps infection. Under short day conditions at 6 wpi, symptoms such as discoloration, leaf curling and increased number axillary leaves are distinctly observed only in CLps infected plants. Severity of symptoms increases in later stages of infection however senescence of plants may also contribute to increased symptoms. Several similarities in pathogen transmission mechanism, latency in incubation and symptom development and disease symptoms demonstrate potential of *Arabidopsis*-BC/CLps as model plant-vector-pathogen system for HLB disease.

Supplementary Information

Table S1.1: Comparison of Ct values generated by SYBR green assay and TaqMan for 19 different tissue samples from different plants

Sample ID	**Biorad IQ5 machine Ct values***	
	SYBR green assay	TaqMan assay
BT583	27.94	28.03
BT584	30.78	30.74
BT585	31.28	29.89
BT586	31.34	29.14
BT587	31.92	31.63
BT588	N/A	N/A
BT589	31.14	30.91
BT590	32.76	30.06
BT591	28.65	29.23
BT592	31.93	31.93
BT593	31.11	30.75
BT594	31.63	32.01
BT595	28.07	28.85
BT596	N/A	N/A
BT597	36.84	37.61
BT598	N/A	35.54
BT602	23.14	23.59
BT603	N/A	N/A
BT604	25.13	25.67

***Ct value is inversely proportional to bacterial titer.**

Calculations for *Candidatus* Liberibacter psyllaurous genome equivalents:

The molecular weight of each plasmid molecule 3476220 (g/mole) was calculated by adding the plasmid size (4000 bp) and insert size (*16S rDNA*, 1267bp) and then multiplying it by 660 (330 da *2 nucleotide/ bp). The weight of a plasmid molecule (5.7724E-18 g/molecule) was calculated by dividing 3476220 g/mole by Avogadro's number. Plasmid molecules per microliter were determined by dividing the concentration of plasmid (118ng/µl) by 5.7724E-18 g/molecule. The number of plasmid molecules per microliter in stock solution was 2.04E+10. Each plasmid molecule would correspond to one *16S rDNA* and with 3 copies of 16S rDNA in CLps genome; the total number of genome equivalents is determined by dividing 2.04E+10 by 3 for stock solution. Table S1.2 shows Ct values and corresponding copy numbers.

Table S1.2. Relationship between Ct values and the number of CLps genomes in a dilution series of qPCR reactions.

Dilutions	CLps genome equivalents in 2 µl *	Log of CLps genome equivalents	Cycle threshold values (Ct)
Stock (X)	13628074073		
0.1X	1.36E+09	9.13	14.83
0.01X	1.36E+08	8.13	18.56
0.001X	1.36E+07	7.13	21.84
0.0001X	1.36E+06	6.13	25.51
0.00001X	1.36E+05	5.13	28.97
0.000001X	1.36E+04	4.13	32.44

2 µl of DNA was used in single PCR reaction

Copy number calculations for *ACT2* gene:

Mass of DNA per genome in grams is calculated by multiplying genome size by mass of DNA

Mass of DNA per genome in grams =Haploid genome size multiplied by average molecular weight of DNA/ Avogadro's number

Arabidopsis genome size (haploid) = 135000000, Mass of DNA= 660g/mole ÷ 6.03E+23 molecules/mole

Mass of DNA per *Arabidopsis* genome in grams = 135000000 X (660g/mole ÷ 6.03E+23 molecules/mole) = 1.48E-13 grams or 0.148 picograms

0.148 picogram (pg) contains one *ACT2* gene

Concentrations of genomic DNA required for the *ACT2* copy numbers of interest in 2µl of PCR volume of template DNA was determined as follows:

Table S1.3: Calculation of concentration of DNA for desired copy numbers of *ACT2*

Copy numbers (A)		**Mass of gDNA needed (pg)**	**PCR volume (µl)**	**Concentration of gDNA in pg per µl (B)**
300000	Multiply by 0.148	44328.35821	2	22164.1791
30000		4432.835821	2	2216.41791
3000		443.2835821	2	221.641791
300		44.32835821	2	22.1641791
30		4.432835821	2	2.21641791

To determine the number of *ACT2* copies and corresponding plant cell numbers, the formula, C1*V1 = C2*V2 was used. The stock concentration of standard *Arabidopsis* genomic DNA was 330ng/µl. The starting concentration C1 was 330000 pg/ µl. Each dilution had a final volume (V2) of 100 µl.

Table S1.4: Number of copies of *ACT2* and plant cells in stock solution and 10 fold dilutions of stock.

***ACT2* DNA conc. in picograms per µl (C)**	**Copies of *ACT2* gene in 2 µl of PCR (D)***	**Plant cells (E)**	**Corresponding Ct values from dilution series**	**Log of plant cells**
330000	4466666.67	2233333.33	19.36	6.35
33000	446666.67	223333.33	22.46	5.35
3300	44666.67	22333.33	26.12	4.35
330	4466.67	2233.333	30.97	3.35
33	446.67	223.33	32.79	2.35

(*Table S1.4 calculations using the formula D* = [C X A (from table S1.3) ÷ B], E = [D / 2]

Table S1.5: Variation in bacterial genomes/plant cell in defense compromised and wild type lines.

Lines	**Average**	**Median**	**Standard deviation**
NahG	48.83	24.57	53.47
npr1-1	67.12	24.74	93.37
ein2-5	43.75	41.77	34.94
pad4	5.43	8.14	6.19
Ler-1	21.68	26.41	18.53
Col-0	97.14	19.17	171.41
coi-1	43.37	41.44	35.47
Col-0	544.35	66.83	860.89

CHAPTER 2: Transcriptional and phenotypic responses of *Arabidopsis thaliana* wild type Columbia-0 and a transgenic, salicylic acid deficient line, *NahG*, to *Candidatus* Liberibacter psyllaurous infection when transmitted by *Bactericera cockerelli.*

SUMMARY:

Understanding the mechanism of disease development at the molecular level can provide crucial information to design a control or curative measure for the disease. Studies on transcriptional analysis of Huanglongbing (HLB) infected citrus have revealed significant changes in the genes involved in processes such as defense, carbohydrate metabolism, cellular transport and organization. Several similarities including the pathogens *16S rDNA* sequences, their mode of transmission and symptomology exist between HLB and Psyllid Yellows. "Psyllid yellows" is a solanaceous disease associated with *Candidatus* Liberibacter psyllaurous (CLps) and *16S rDNA* sequence comparisons indicate that CLps is closely related to the Huanglongbing pathogen. Our objective was to investigate *Arabidopsis* responses to CLps infection and determine similarities and differences with HLB disease, as a model for research on citrus. In this study, I report transcriptional and phenotypic changes of *Arabidopsis thaliana* in response to CLps infection via psyllid transmission. Wild type Col-0 and the salicylic acid deficient *NahG* line were infested with psyllids positive and negative for CLps and differentially expressed transcripts in response to CLps-infection were determined using a microarray. At 8 weeks post-infestation (wpi), *NahG* line had higher percentage of CLps infected

plants in comparison to Col-0 however; the bacterial genome equivalents /plant cell was higher in Col-0. Analysis of phenotype changes in leaf and stem development indicated that CLps-infected plants were significantly slower in development and growth than the non-infected plants. A notable symptom present only in infected plants was a profuse growth of small size axillary leaves, especially distinct on *NahG* plants. Microarray analysis at 8 wpi identified 130 and 52 total differentially expressed genes (DEGS) in response to CLps infection in Col-0 and *NahG* lines respectively. In both lines, a majority of the genes induced were involved in various defense pathways or signaling, metabolic and cellular processes. Among the notable genes that showed significant up-regulation in response to CLps infection were the WRKY transcription factors, pathogenesis related proteins and oxidative stress related genes. Also, included were several developmental growth related genes encoding AGL42, AGL8 and MADS box transcription factors involved in floral transition were significantly down-regulated. No gene was significantly and differentially regulated in response to psyllid feeding at 8 wpi. Comparison to differentially expressed genes in HLB infected citrus indicates similarities in the up-regulation of several defense genes encoding peroxidase superfamily protein, Chitinase, Osmotin34, Kunitz trypsin inhibitor 1 and pathogenesis related proteins

ABBREVIATIONS:

DEGs -differentially expressed genes, CLps – Candidatus Liberibacter psyllaurous, SAR – Systemic acquired resistances, SA- Salicylic acid, JA-Jasmonic acid, BC – Bactericera cockerelli, wpi- weeks post infestation.

INTRODUCTION

Molecular and genetic dissection of plant's defense responses to pathogen is highly useful to develop strategies that can help control or possibly eliminate disease. Plants respond to various pathogens and pests using different defense pathways that work in a highly coordinated manner. These responses are typically reflected by the changes in plant growth, development as well as changes in gene expression patterns as a consequence of pathogen infection. Huanglongbing is a bacterial disease that affects many citrus species and causes major damage to the plant's growth and productivity. This century-old disease is considered as one of the most destructive diseases of the citrus industry, mainly due to lack of an effective curative or control measure. The development of such measures is hindered by the challenges such as inability to culture the HLB pathogen in artificial media, limited genomic resources, long generation time, incompatibility, apomixis and other issues associated with the citrus/HLB system in culture. These challenges limit a rapid understanding of plant-pathogen interactions and development of a control/curative measure for the pathogen in a time effective manner. The goal of this study is to investigate the potential of using psyllid yellows, a solanaceous disease associated with a pathogen related to the causal agent of HLB, and *Arabidopsis thaliana* as a model plant-pathosystem in HLB related research.

Psyllid Yellows

Psyllid yellows was discovered in potato plants as a yellowing condition thought to result from a toxin injected by hemipteren psyllid *Bactericera cockerelli* (BC) (Blood, 1933b; Blood et al., 1933a; Richards, 1931). However, recent studies show that psyllid yellows is associated with a bacteria known as *Candidatus* Liberibacte*r* psyllaurous transmitted by a psyllid BC (Hansen et al., 2008). The CLps bacteria is a gram negative, sieve tube inhabiting alpha-proteobacteria and is currently uncultivable in artificial media. Hansen's study (Hansen et al., 2008) showed horizontal and vertical transmission of CLps into BC. The *Bactericera cockerelli* psyllid can multiply on a range of solanaceous plant species such as *Solanum lycopersicum* (tomato), *Solanum tuberosum* (potato), and *Capsicum annum* (pepper), so it is very likely that most of its host plants can also be hosts to the CLps pathogen. The CLps-infected tomato plants display symptoms such as leaf discoloration or yellowing, purpling along mid-veins, shorter internodes, leaf cupping, lower fruit production or small size fruits and overall stunted growth of plants (Cranshaw, 1994; Liefting et al., 2009; Pletsch, 1947). The CLps Infection in tomato plants can also lead to early senescence and premature death (Cranshaw, 1994; Liefting et al., 2009; Pletsch, 1947)· Studies indicate that psyllid infestation can cause substantial damage to the solanaceous plants, however the plants infested with psyllids and positive for CLps can suffer more severe damage and even sudden death (Sengoda et al., 2010). *Candidatus* Liberibacter psyllaurous is also referred

as *Candidatus* Liberibacter solanacearum (Liefting et al., 2009) and has been associated with another disease of potatoes known as zebra chip (Munyaneza et al., 2007a, 2007b). This disease has similarities with psyllid yellows in regards to symptoms displayed by the above ground plant parts, however unlike psyllid yellows of potato, zebra chip affects potato tubers which display striped pattern of necrosis (Sengoda et al., 2010). Both psyllid yellows and zebra chip diseases are associated with *Candidatus* Liberibacter species and cause significant damage to the productivity of solanaceous plants, therefore it is critical to understand the underlying molecular mechanisms of interactions between the transmitted pathogen and plants to develop curative or control measures. Casteel (Casteel et al., 2012) showed that the responses of tomato plants to CLps transmission (and infection) by psyllids and grafting involves a complex pattern of defense gene regulation that was dependent on psyllid stages and CLps inoculum titer. Suppression of defense genes was observed in response to CLps transmission by older psyllids that have higher CLps titer. It is evident from both studies (Casteel et al., 2012; Sengoda et al., 2010), that plants suffer more damages in presence of CLps, and plant responses are manipulated by CLps for the survival of both the psyllid and itself. In our study we investigate the responses of *Arabidopsis* (non-host of BC) to CLps and psyllids separately to understand specific changes caused by CLps infection.

Huanglongbing

A *Candidatus* Liberibacter species associated disease of citrus is known as Huanglongbing, and has many similarities to psyllid yellows and zebra chip diseases. Among several diseases affecting the citrus industry, HLB is considered to be the most destructive (Bové, 2006; Gottwald et al., 2007). HLB has been prevalent in many Asian and African countries where it has caused millions of dollars of damage. In South Vietnam, application of insecticidal sprays to control this vector transmitted disease resulted in HLB incidence of 76% but without sprays disease incidence was 96% in citrus orchards (Gatineau et al., 2006). In a survey of Reunion Island, 65% of plants were found to be infected and became unproductive within 7 years of planting (Aubert et al., 1996). In 2005, HLB was detected for the first time in Florida and now has spread into all the citrus growing counties of Florida (Gottwald, 2010). HLB is associated with three related phloem limited bacterial species *Candidatus* Liberibacter asiaticus (CLas) (Jagoueix et al., 1994), *Candidatus* Liberibacter americanus (CLam) (Texeira et al., 2005b), and *Candidatus* Liberibacter africanus (CLaf) (Jagoueix et al., 1994) transmitted by hemipteren psyllid vectors. All the HLB associated Liberibacter species are gram negative, alpha-proteobacteria, and have been given the status "*Candidatus*" to indicate that they have not been cultured in media. CLas and CLam are transmitted by the psyllid *Diaphorina citri* (Kuwayama) (Capoor et al., 1967; Texeira et al., 2005b) and CLaf is transmitted by *Trioza erytreae* Del Guercio (McClean and Oberholzer, 1965). Besides

psyllid transmission, these bacteria can also spread by graft transmission (Van Vuuren, 1993). Typically, HLB infected trees are asymptomatic at early stages of infection and depending on factors such as growing conditions and age of tree, symptom appearance can vary among citrus genotypes from 6 months to 3 years (Achor et al., 2010; Bove, 2006; Folimonova et al., 2009) Severely infected trees display symptoms such as asymmetric chlorosis or blotchy mottle appearance on leaves, reduced fruit yield, or small, lopsided fruits with bitter fruit juice that often contain aborted seeds (Bassanezi et al., 2009, 2011; McClean and Schwarz, 1970). Various surveys show that HLB affects almost all known citrus varieties and citrus relatives (Bhagabati, 1993; Miyakawa, 1980; Miyakawa and Yuan, 1990). Mandarins, sweet orange, tangerines were found more susceptible in comparison to grapefruits (Bhagabati, 1993; Miyakawa, 1980; Miyakawa and Yuan, 1990). Although studies (Albrecht and Bowman, 2011; Bhagabati, 1993) show some degree of tolerance in species such as *Citrus indica* Tanaka, *Citrus macroptera* Montrouz and US897 (Hybrid of *C. reticulata* 'Cleopatra' × *P. trifoliata*), no variety has been reported as completely resistant to HLB disease. Understanding the disease progression from early to later stages and whether genetic variation in the host can affect disease spread can aid in developing measures to prevent the disease. Several research efforts have been undertaken to understand anatomical, biochemical and molecular changes in HLB disease. Scattered necrotic phloem and massive starch accumulation present uniformly in symptomatic tissues was evident in the anatomical study of HLB infected tissues by Schneider (Schneider, 1968). Microscopic studies reveal

the presence of starch grains in phloem elements, phloem parenchyma, mesophyll, and palisade cells of HLB infected leaves (Etxeberria et al., 2009). Distorted chloroplasts containing large granular starch were observed in HLB infected tissues (Achor et al., 2010; Etxeberria et al., 2009). HLB related decline of tree health is linked to the inefficient transport of photoassimilate in phloem due to phloem plugging by starch, callose and phloem specific protein PP2 accumulation (Achor et al., 2010; Kim et al., 2009). However, interestingly anatomical studies of roots show that CLas infection in roots results in the loss of roots and this loss is not due to phloem plugging or carbohydrate starvation as it happens in stems (Johnson et al., 2014). These anatomical observations were supported by several gene expression studies of HLB infected citrus trees (Albrecht and Bowman, 2008, 2011, 2012b; Kim et al., 2009; Mafra et al., 2013).

HLB related gene expression studies in citrus:

Candidatus Liberibacter species associated with Huanglongbing induce a range of transcriptional and biochemical changes in different citrus varieties (Albrecht and Bowman, 2011; Fan et al., 2012; Kim et al., 2009; Mafra et al., 2013; Martinelli et al., 2012; Nwugo et al., 2013; Albrecht and Bowman, 2008). In 2008, the first study of gene expression profiling of HLB infected citrus provided a detailed insight into the responses of sweet orange to CLas infection for two time points, 5-9 weeks post inoculation and 13-17 weeks post inoculation (Albrecht and Bowman, 2008). The study (Albrecht and Bowman, 2008) showed that 33-44%, 16%, 12%, 13% and 2-6% of differentially

expressed genes (DEGs) were involved in cellular/metabolic processes, response to abiotic or biotic stimulus or to stress, cell organization or biogenesis, transport and signal transduction and transcription respectively. At 13-17 weeks time point, 27-50% of DEGs encode proteins involved in the endo-membrane system and a significant percentage (26%) of down-regulated genes were chloroplast related (Albrecht and Bowman, 2008). Some defense related genes were up-regulated at different time points following the infection. For example, alcohol:$NADP^+$-oxidoreductase, associated with hypersensitive response, was up-regulated 2-3 fold at 5-9 weeks post inoculation, whereas bacterial peroxidase precursor, lipooxygenase and heat shock protein genes were significantly up-regulated at 13-17 weeks post inoculation by more than 4 fold. Among significantly down-regulated genes were basic endochitinase, chlorophyll a/b binding family protein, and PSII 5KDa protein. Several genes involved in carbohydrate metabolism such as ADP-glucose pyrophosphorylase, starch synthase, starch phosphorylase, and α-amylase were significantly up-regulated whereas β-amylase, sucrose phosphate synthase were significantly down-regulated. Notably, in this study the higher expression of a phloem specific protein known as *PP2* was predicted to have a role in symptom development. In 2009, a different transcriptional study (Kim et al., 2009) showed a similar expression profile of genes to that reported by Albrecht and Bowman (2008). It confirmed changes in the expression of genes such as chitinase, pathogenesis related proteins, miraculin like proteins, *PP2* and genes involved in carbohydrate metabolism such as ADP-glucose pyrophosphorylase, β-glucosidase. Some

studies have compared the gene expression changes between HLB tolerant and susceptible citrus species (Albrecht and Bowman, 2011; Fan et al., 2012) to determine defense responses to HLB infection. A time course (5 wpi, 17 wpi and 27 wpi) comparative analysis of transcriptional profiles in rough lemon (tolerant citrus) and sweet orange (susceptible citrus) revealed some interesting HLB related changes in the gene expression of these varieties (Fan et al., 2012). In this study, not many DEGs were detected at 5 wpi, however, at 17 wpi, 683 and 181 genes were differentially expressed in rough lemon and sweet orange respectively. At 27 wpi, more genes were differentially expressed in sweet orange than in rough lemon indicating delayed response to CLas infection. Similar to other HLB-related gene expression studies, more genes were up-regulated and fewer genes were down-regulated in this study (Fan et al., 2012). A difference in HLB related DEGs profiles between the two varieties was down regulation of cell wall metabolism genes in lemon at 5 wpi followed by up-regulation of these genes at 27 wpi, whereas exactly opposite changes were detected in sweet orange(Fan et al., 2012). Another notable difference was in hormone signaling pathway regulation in these varieties. Abscisic acid related genes showed up-regulation in rough lemon whereas in sweet orange, genes involved in the ethylene pathway showed up-regulation. A few key changes such as higher fold induction of callose degrading enzyme β-1-3 GLUCANASE in rough lemon than sweet orange, lower transcript levels of the phloem plugging protein known as PP2 in rough lemon than sweet orange and correspondingly less damage to phloem transport activity in rough lemon than sweet

orange were suggested as the factors contributing to the increased tolerance of rough lemon. A comparative study between US897 (HLB tolerant, hybrid of *C. reticulata* 'Cleopatra' × *P. trifoliata)* and Cleopatra mandarin (HLB susceptible) was conducted to dissect the mechanism of tolerance in US897 (Albrecht and Bowman, 2012a). HLB tolerance of US897 results from higher fold induction of defense related genes such as *PATHOGENESIS RELATED GENE 5* (*PR5)*, *PLANT DEFENSIN 2.2* (*PDF2.2*), *OSMOTIN34* (*OSM34*) and less induction of *PP2* gene than in the susceptible Cleopatra mandarin. All these HLB related gene expression studies show that the timing and fold induction of defense related genes and regulation of carbohydrate metabolism are correlated to symptom development. The plant's response to the pathogen such as callose deposition and accumulation of PP2 protein may cause damage to the phloem and thereby affect transport processes. Comparison of psyllid yellows and Huanglongbing reveal similarities in mechanism of pathogen transmission (grafting and insect vectors), symptoms and most importantly in phylogenetic relationships of the pathogens (*16S rDNA* sequence shows 97% similarity between CLps and CLas, (Hansen et al., 2008)). Such similarities between plant-pathosystems provide evidence that PY has a potential as a model system for studying HLB related interactions. Such model system studies are bolstered if the host plant is a well-studied plant species such as *Arabidopsis thaliana*.

Arabidopsis is the most extensively studied plant species, predominantly due to its characteristic features such as small size, rapid life cycle, abundant seed production, ease in manipulation and replication. Its numerous genetic and genomics resources offer significant advantage for studies of any molecular processes compared to other plants. It has been used as model plant in many developmental and defense studies.

Here I report a study of changes in gene expression and phenotype of *Arabidopsis thaliana* to *Candidatus* Liberibacter psyllaurous infection. Two lines were used in this study a defense compromised *NahG* line and wild type Col-0 line. The *NahG* line is a transgenic Col-0, which is transformed with a gene encoding salicylate hydroxylase. The salicylate hydroxylase enzyme converts salicylic acid into catechol and as a result; the plant is deficient in SA and cannot trigger adequate down-stream defense responses. Both *NahG* and wild type Col-0 lines were analyzed for phenotypic and transcriptional changes. These changes were compared among and across four treatments for each line. The four treatments were non-infested plants, plants infested with CLps-negative psyllids, plants infested with CLps positive psyllids but CLps negative and asymptomatic, and plants infested with CLps positive psyllids and CLps positive and symptomatic. Transcriptome (transcript accumulation) analysis using a microarray at 8 wpi and using qRT-PCR at 3 wpi (only candidate genes) were carried out to determine responses induced by CLps infection. Phenotypic changes in leaves, stems, flowers and overall growth of plants were documented from 3 to 8 wpi. All infected plants displayed strong

CLps infection related symptoms and were significantly slower in growth and development. Transcriptional analysis showed activation of genes involved in basal defense, but these responses were inadequate for complete resistance to CLps.

MATERIALS AND METHODS:

Plant material

Seeds for *Arabidopsis thaliana NahG* (salicylic acid deficient) and Col-0 (wild type) homozygous lines provided by Dr. Thomas Eulgem (University of California, Riverside) were stratified for 72 hrs at 4^{o}C in pesticide/fungicide free soil for uniform germination. All plants were grown under short day conditions (8 hrs day at 23C /16 hrs night at 22C) in an environmental growth chamber. At 4-weeks stage, one plant per pot was infested with 10 nymphs (mixture of 3^{rd}-5^{th} instar stage nymphs) and covered with an organza bag to prevent psyllid escape. All infested and non-infested plants were placed in separate bugdorms (BioQuip products, 1462W). Plants in each tray were randomly rearranged twice a week and each bugdorm was rotated twice a week in the growth chamber. At 3 weeks post infestation, all plants were assigned to three blocks that differed by 24 hours in the time of tissue collection and phenotype observations. Each block contained 36 plants of each line: 12 non-infested, 12 plants infested with CLps negative BCs, and 12 plants infested with CLps positive BCs. Random rotation of plants within trays and sequential rotation of each block continued throughout experiment to avoid location-related effects on plant phenotype.

Insect Material:

All colonies of *Bactericera cockerelli* (BC, tomato psyllid) were maintained on host *Solanum lycopersicum* var. Moneymaker plant (Source: Dr. Trumble, Department of Entomology, University of California, Riverside) at 21-26^{o}C and 40-60% relative humidity. One colony-contained psyllids tested positive for *Candidatus* Liberibacter psyllaurous (90-98% of nymphs and adults were detected positive for *Candidatus* Liberibacter psyllaurous) and another colony contained psyllids tested negative for CLps. These colonies were located in different buildings and movement between the CLps-positive colony and the CLps-negative colony was avoided at all times.

Infestation of plants:

Prior to infestation of plants, all non-infested plants were covered with organza bags and placed in a separate bugdorm. Plants selected for infestation were at the same growth stage (stage number 1.06, 6 ±1 rosette leaves). A mixture of late instar stage nymphs was used for infesting and all nymphs were selected from symptomatic tomato plants. Each nymph selected based on its size and color was carefully picked with a small paintbrush and placed on an *Arabidopsis* leaf. Care was taken to avoid breakage of the psyllid stylet by choosing psyllids that were not feeding on leaves. After placing 10 nymphs, each plant was covered with an organza mesh bag to restrict psyllid movement to other plants. These plants were covered until 2-3 weeks post infestation. Infestation

with CLps negative nymphs was carried out before the infestation with CLps positive nymphs for both lines.

DNA extraction and Real time quantitative PCR for CLps detection:

For the microarray experiment, leaves (rosette and cauline) and other tissue samples were harvested at 8 wpi for DNA extraction to detect CLps. In a separate experiment to validate microarray results, leaves (rosette and cauline) were harvested at 3 wpi and 8 wpi for DNA extraction to detect CLps. Individual tissues were transferred into 2 ml tubes containing 2.5 mm zirconium beads (Research Products International Corp.) with 700 µl of extraction buffer (100 mM Tris, (pH 7.5) 500 mM NaCl, 50 mM EDTA, 2.5% SDS). Samples were homogenized for 60 seconds in a MP Biomedicals FastPrep®-24 at a setting of speed 6.5m/s and time 60 seconds. After centrifugation, isopropanol and 0.2 M potassium acetate were added to the supernatant for DNA precipitation. All samples were incubated overnight at -20°C and the precipitated DNA pellets were cleaned with 70% ethanol. Purified DNA was dissolved in 100 µl of sterile water and stored at 4°C. DNA samples with A260/A280 ratios between 1.7-2.00 were used for pathogen detection by qPCR.

TaqMan based quantitative PCR assay for CLps detection (qPCR):

All qPCR reactions were set up in 96 well plates (USA Scientific) with each reaction mixture (25 µl) containing 1X reaction buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, pH 8.3, New England Biolabs), 250 µM dNTPs (Promega), 2.5 units of *Taq* DNA polymerase (New England Biolabs), 0.25 µM each of primers Les 33, Les 34, Les 37, Les 38, 0.15 µM each of Liberibacter and *ACT2* probes (Table 2.1), water added to bring the volume to 23µl, and 2µl of template. All reactions were performed in a Bio-Rad iQ5 thermal cycler with cycling conditions 95.0 °C for 10 min, followed by 40 cycles of 95.0 °C for 30 seconds and 58.0 °C for 55 seconds. In all qPCR assays, 2 to 4 non-template controls (NTC), and *Ca*. L. psyllaurous-positive standard DNA were included. All NTC controls were randomly placed to detect cross contamination among reactions. The number of CLps genomes and plant cells were determined using the standard curves generated for CLps *16S rDNA* (EU812559) and *Arabidopsis ACT2* gene. The standard curve for CLps *16S* rDNA was generated with Ct values of 10-fold serial dilutions of plasmid DNA containing *16S* rDNA and the corresponding log of copy numbers of target DNA. Standard curve of *Arabidopsis ACT2* gene was generated using Ct values of 10 fold serial dilutions of a plant sample (# GT711) with DNA concentration (330ng/µl) and corresponding log of copy numbers of target DNA. Copy number calculations for both genes are described in supplementary information of Chapter 1 (TableS1.2-Table S1.4).

Table 2.1: Primers and probes used in real time quantitative PCR for CLps amplification.

Gene ID*	Target genes	Target Organism	Primer ID used in this study	Sequences	Reference
EU812559	*16S rDNA*	CLps	Les33	TCGAGCGCTTATTTTTAATAGG	(Hansen et al., 2008)
L22532	*16S rDNA*	CLas	Les34	TGCGTTATCCCGTAGAAAAAGGTAG	(Li et al., 2006)
821411	ACTIN 2	*Arabidopsis*	Les37	GTTGGGATGAACCAGAAGGA	This study
821411	ACTIN 2	*Arabidopsis*	Les 38	GGCCTTTGGGTTAAGAGGAG	This study
L22532	*16S rDNA*	Liberibacter	Les39	5' FAM-AGACGGG TGAG TAACGCG-3' BHQ1	(Li et al., 2006)
821411	ACTIN 2	*Arabidopsis*	Les41 ACT2-probe	5' Cy5-TTGCTCCTGAAGAGCAC CCTGTTCTT-BHQ3 - 3'	This study

* NCBI gene ID or GenBank Accession ID number, CLas – *Candidatus* Liberibacter asiaticus**.**

Phenotype data:

All plants were visually monitored for phenotype changes. Throughout the experiment all plants were photographed weekly except 7 wpi (due to time limitation). Plant growth and development including leaf number, color, and stem/floral development were noted weekly for all plants from 3 wpi until 8 wpi. Data were analyzed using chi-square tests for frequency data and, for measurement data collected on each plant over time, repeated measures analysis of variance using GLM (SAS 9.3).

RNA isolation and quality assessment for microarray:

Leaf samples from all plants were divided into two parts, one part to be used for CLps detection and other to be used for RNA isolation. Total RNA was isolated from cauline/axillary leaves using TRIzol® (Life Technologies) following the manufacturer's

instructions. Concentration and quality of all RNA samples were assessed from the absorbance readings at 260 nm and 280 nm using a Nanodrop ND2000 spectrophotometer (Thermo Scientific) and Bioanalyzer (Agilent 2100). For each line, three samples were pooled from each treatment from each block of experiment. Each pooled sample was then assessed with the spectrophotometer (NanoDrop 2000, Thermo Scientific) and Bioanalyzer (Agilent 2100). Pools with concentration more than 150 ng/µl and A260/A280 and A260/A230 ratios more than 1.8 were selected for microarray hybridization.

Microarray preparation and data analysis:

For each replicate, the pooled RNA sample was hybridized to ATH1 genome array gene chip (Affymeterix) at Institute for Integrative Genome Biology, core facility (University of California, Riverside). Three biological replicates for each treatment and each line were analyzed. Bioconductor packages in R programming environment were used for processing and analyzing all the data. Raw and log transformed images were analyzed for spatial bias by affy package (affyPLM) of Bioconductor (Bolstad, 2004). Raw data was also analyzed for quality assessment, then preprocessed and normalized using the RMA (robust multichip averaging) algorithm (Irizarry et al., 2003) and differentially expressed genes were identified using the LIMMA package (Smyth, 2005). Adjusted p-values for multiple testing were calculated by Benjamini and Hochberg method (Benjamini and Hochberg, 1995). Clustering of differentially expressed genes into a

defined number of clusters was performed using the fuzzy K-means clustering algorithm by function fanny of Cluster package in R environment (Maechler et al., 2014). The gene ontology term enrichment analysis was performed with clustered genes and significance of GO analysis was determined by hypergeometric probability distribution function "GOHyperGAll" in R environment (Horan et al., 2008). Venn diagrams were also created to identify DEGs commonly expressed in *NahG* and Col-0 lines in response to CLps infection using Venny tool (Oliveros, 2007). Functional annotations and descriptions of differentially expressed gene were gathered from TAIR (Garcia-Hernandez et al., 2002) and related published literature.

Reverse Transcription Quantitative real time PCR (qRT-PCR)

Fifteen candidate genes were selected based on their expression levels detected in microarray for both Col-0 and *NahG* lines, as well as the expression of their citrus orthologs in HLB infected citrus trees. Gene specific qRT-PCR primers (Supplementary table 2.1S) were designed using Primer3 (Koressaar and Remm, 2007), PrimerQuest software (Integrated DNA Technologies) and Primer Blast (NCBI). For each gene, multiple primer pairs were designed using software and the optimal pair was selected based on structure and specificity. Primer specificity to the gene sequence was confirmed by using Primer Blast (NCBI). DNAse treated RNA was reverse transcribed to cDNA by M-MuLV Reverse Transcriptase protocol (New England Biolabs). All qRT-PCR reactions were set up using SensiMix™ SYBR® & Fluorescein Kit (Bioline) in a CFX 96 real

time thermocycler (Bio-Rad). PCR cycling conditions for all the primer pairs were 95.0 °C for 10 minutes, followed by 40 cycles of 95.0 °C for 30 seconds and 60.0 °C for 55 seconds. Melting curves of PCR amplicons were obtained with temperatures ranging from 65°C to 95°C with a 0.5°C increase in temperature every 10 seconds. Four housekeeping genes were tested for their stability across the treatments *UBQ10* (AT4G05320), *ACT2* (AT3G18780), *EF1A* (AT5G60390), and *UBP22* (AT5G10790). A stable house keeping gene was selected using BestKeeper Excel based tool (Pfaffl et al., 2004). Fold change in expression was determined by using *UBQ10* (AT4G05320) as the reference gene by Pfaffl method (Pfaffl, 2001).

RESULTS

Systemic infection of *Candidatus* Liberibacter psyllaurous in *NahG* and Col-0 plants

Multiple tissue samples (leaves and stems) collected from all plants of all treatment-groups (Fig 2.1) were tested for CLps infection at 8 wpi by qPCR using CLps specific primers. No CLps positive plants were detected among plants that were not infested with psyllids, or in those infested with CLps-negative psyllids.

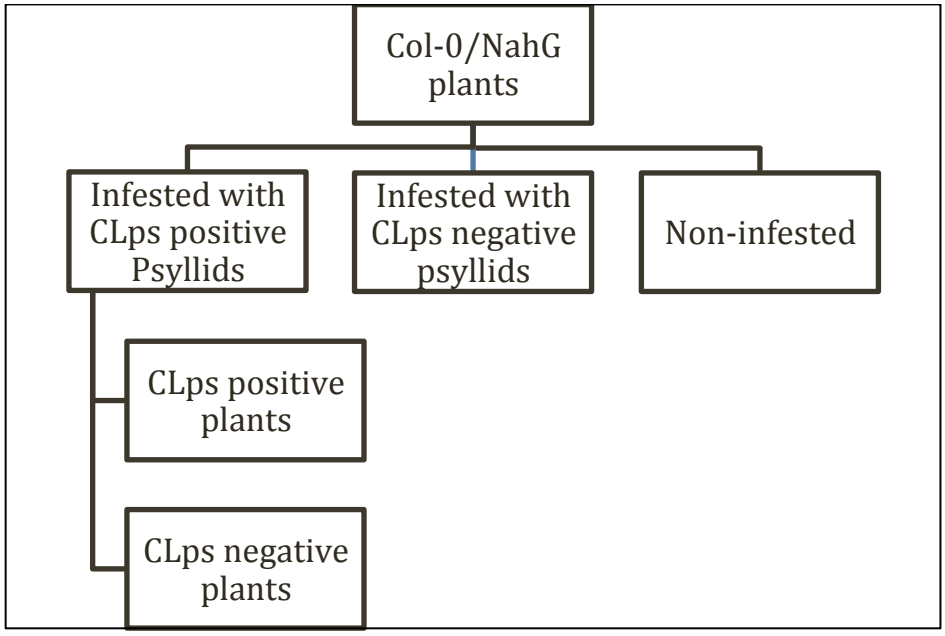

Figure 2.1: Flowchart illustrating different treatment-groups of *NahG* and Col-0 plants in a block of microarray experiment.

Among the plants infested with CLps-positive psyllids, the percentages of CLps positive *Arabidopsis* plants were 25.0 and 36.1 for wild type Col-0 and *NahG* lines respectively, a difference not significant by chi-square test (Fig 2.2A, Criteria: Stringent cut off Ct value of CLps in plant>30). The number of bacterial genomes per plant cell was calculated for

all the tissue samples such as cauline leaves, rosette leaves, and stems using standard curve created by serial dilutions of CLps DNA and plant DNA (Supplementary tables of Chapter 1, S1.2-S1.5). The average number of CLps genome equivalents per plant cell was highly variable and did not differ significantly between lines for any of the tissue types (rosette leaves, stems, and cauline leaves, Fig 2.2, Student's t-test, p value>0.05). For the *NahG* line, the CLps genome equivalents per plant cell in cauline leaves ranged from 3 to 72, in rosette leaves ranged from 0.7 to 64 and in stems ranged from 1.5 to 45. For Col-0, the CLps genome equivalents per plant cell in cauline leaves ranged from 0.48 to 83, rosette leaves ranged from 2 to 21 and in stems ranged from 0.1 to 29. Analysis of CLps genome equivalents/plant cell for different tissue type within each line indicates no statistically significant difference ($p>0.05$,One way ANOVA) for both Col-0 and *NahG* line.

Candidatus Liberibacter psyllaurous infection affects _Arabidopsis thaliana_ growth and induces distinct phenotype changes.

All plants were monitored for phenotypic changes every week. All the observations of changes in leaves; stems and floral tissues development were documented for each plant. No visually distinct changes were observed until 4 wpi. These observations were consistent with latency in symptom development as observed in previous experiments. At 5 wpi, discoloration of leaves on few CLps positive plants was noticed, however no other distinct symptoms were observed. At 6 wpi, several plants infested with CLps

positive psyllids showed distinct discoloration, leaf curling and clustering of small size leaves in the center of the rosette. By 7 wpi and 8 wpi, many CLps positive plants showed symptomatic phenotype such as discolored or purple leaves, excessive small axillary leaves and clusters of small cauline leaves. Fig 2.3A shows the phenotype transition from 3 wpi to 8 wpi of Col-0 plants detected positive and negative for CLps in different treatments. Fig 2.3 B shows the phenotype transition of *NahG* plants from 3 wpi to 8 wpi detected positive and negative for CLps in different treatments. The comparative analysis of several symptoms such as discoloration, leaf curling, stem development, rosette leaf and axillary leaf numbers show that overall plant growth was significantly affected by CLps infection. The number of rosette leaves was significantly less in CLps positive plants than CLps negative plants in both lines by 8 weeks post-infestation. From 3 wpi to 8 wpi, the infested plants had a significantly lower number of rosette leaves than non-infested plants, irrespective of genetic background (Fig 2.4, Repeated measures ANOVA, $p<0.05$). At 8 wpi, the plants detected positive for CLps had a significantly lower number of rosette leaves than non-infested plants and plants detected negative for CLps infection (Fig 2.4, Repeated measures ANOVA, $p<0.05$).

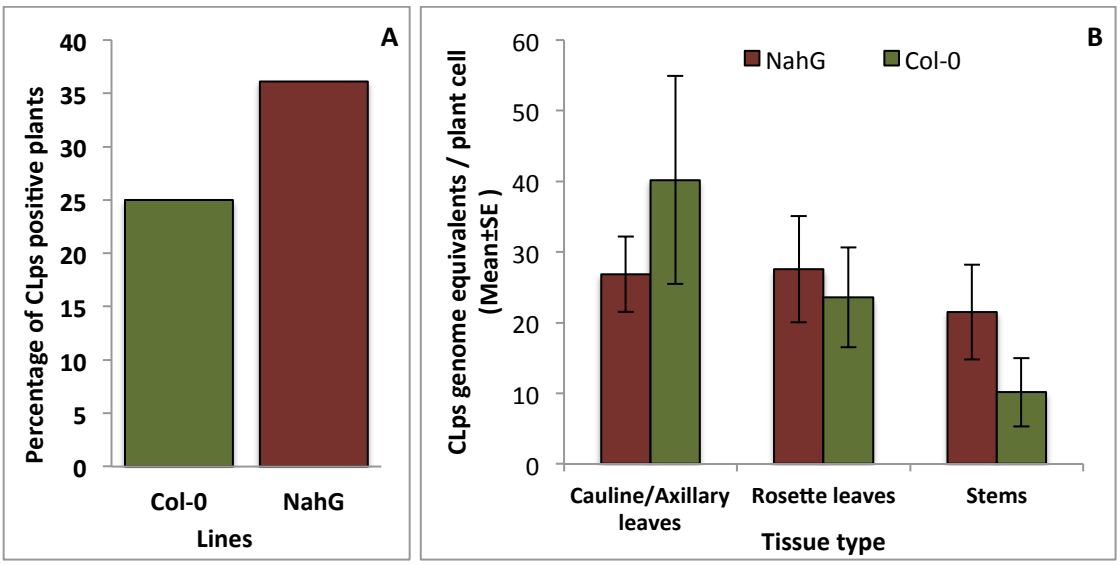

Figure 2.2 A-B: *Candidatus* Liberibacter psyllaurous infection in *NahG* and Col-0 lines infested with 10 psyllid nymphs from CLps positive tomato plants.

A) Percentage of *Candidatus* Liberibacter psyllaurous positive plants in Col-0 (green bar) and *NahG* (brown bar), Chi-Square test, $p>0.05$ **B)** Average number of CLps genome equivalents / plant cell in different tissue types in Col-0 and *NahG* at 8 wpi. Data represents mean of CLps genome equivalents with standard error bars. No statistical significance was detected between lines ($p>0.05$, Student t-test) and within lines ($p>0.05$, one-way ANOVA) for bacterial genomes / plant cell in different tissue types.

Note: *Only 2 plants of Col-0 line had developed stems by 8 wpi.*

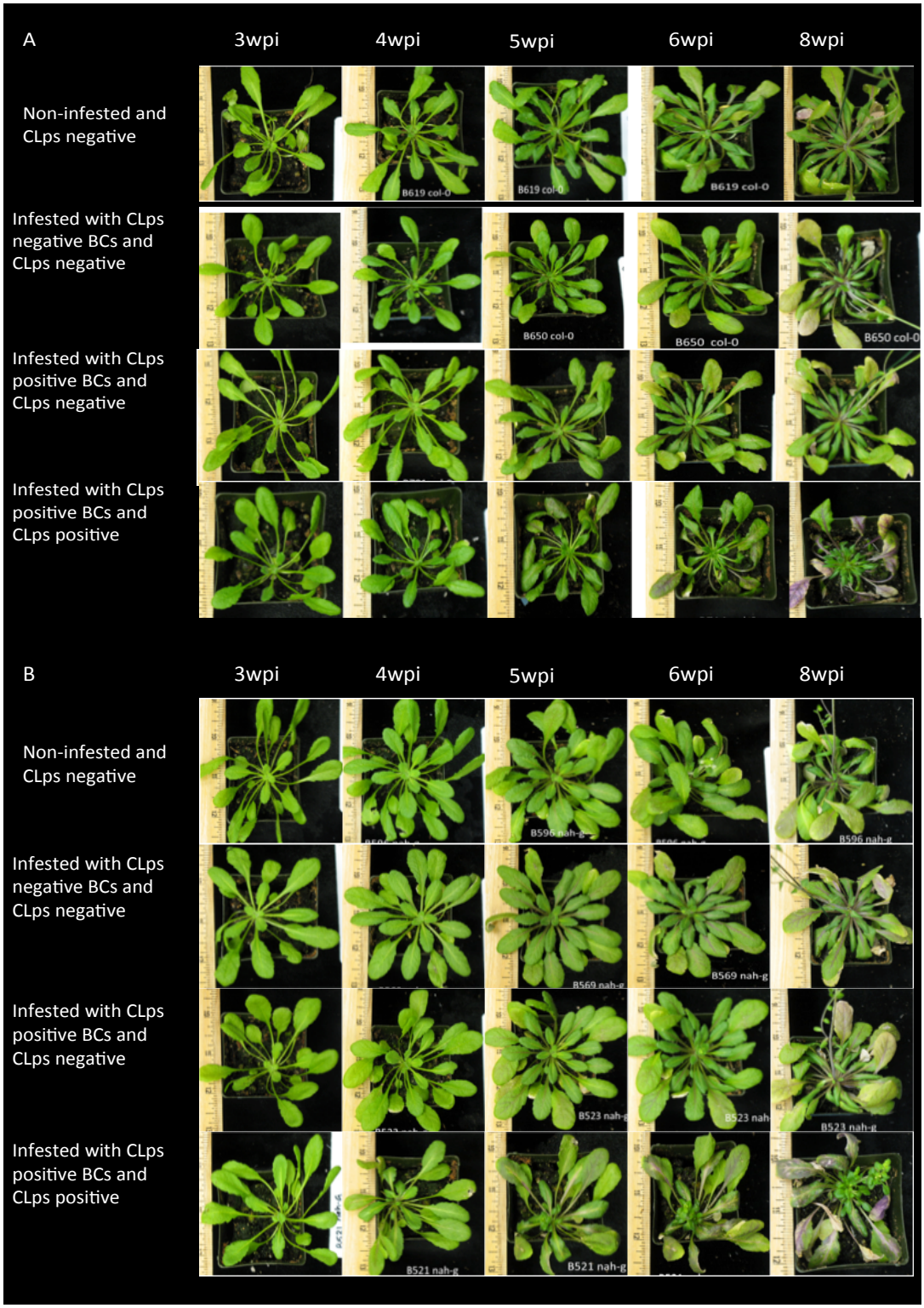

Figure 2.3: Phenotype transition from 3 wpi to 8 wpi in different treatments of Col-0 (2.3A,top) and *NahG* (2.3B, bottom)

Analysis of effect of treatments within Col-0 line showed no significant difference in the number of rosette leaves between CLps negative plants (non-infested, infested with negative BCs, infested with positive BCs and CLps negative) and CLps positive plants until 7 wpi (Fig2.4A). However at 8 wpi, CLps positive plants had significantly lower number of rosette leaves in comparison to other treatments (Fig2.4A, repeated measures ANOVA, $p<0.05$). In the *NahG* line, plants detected positive for CLps infection had consistently lower numbers of rosette leaves than CLps negative plants (non-infested, infested with negative BCs, Infested with positive BCs but CLps negative) and significantly lower numbers from 6 wpi-8 wpi (Fig2.4B). Comparison of *NahG* and Col-0 CLps positive plants indicate no significant difference in the number of rosette leaves (Unpaired t-test, $p>0.05$). The development of stem and its growth was monitored in all plants for both lines. Frequency analysis of plants with stems indicated no significant effect of CLps infection or psyllid feeding on stem development in the *NahG* line (Fig 2.5B) whereas in Col-0 only 22% of infected plants had developed stems in comparison to 84% in non-infected plants. The significant difference (Chi Square test, $p<0.05$) in frequency of plants with stems between the CLps infected and non-infected groups indicates that the pathogen interferes with plant growth in Col-0. Stem length was measured and compared for all plants from 6 wpi-8 wpi and irrespective of genetic background all the CLps positive plants had significantly shorter stems compared to other treatments at 8 wpi (Fig2.5C and D, repeated measures ANOVA $p<0.05$).

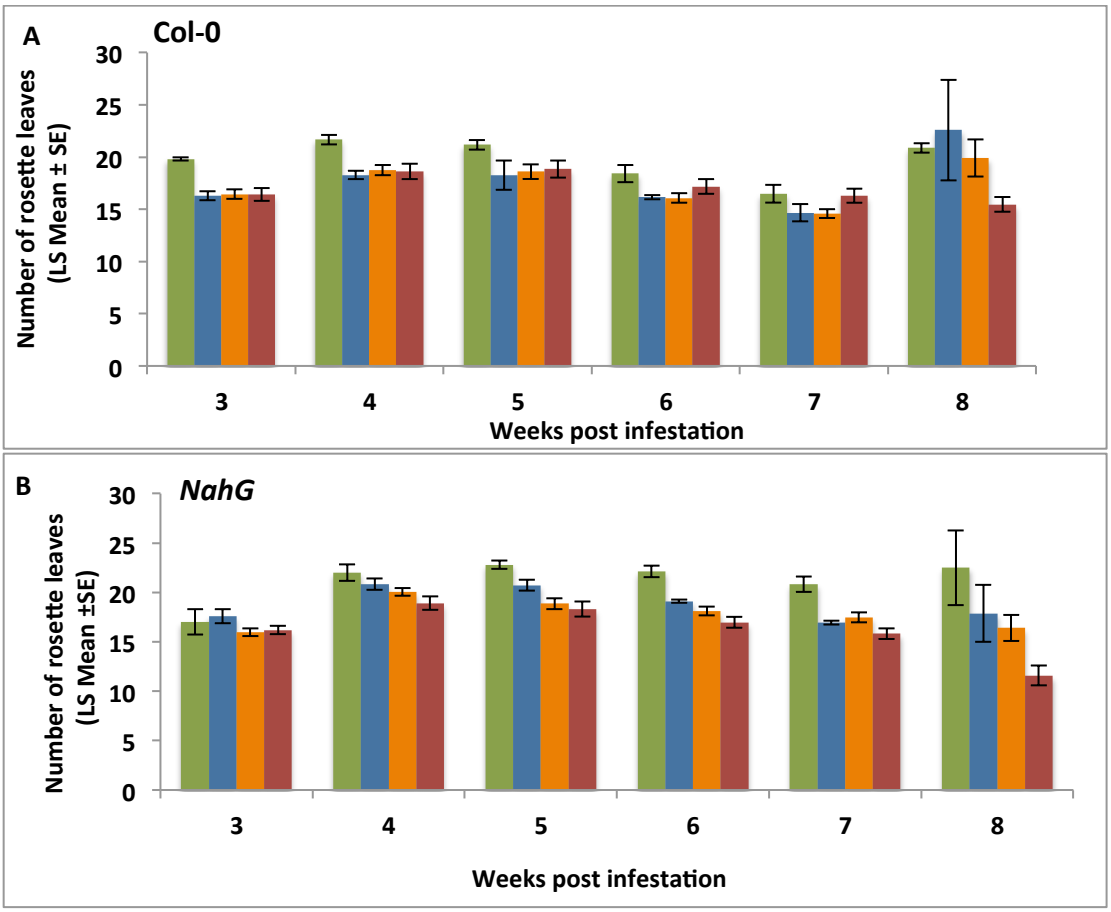

Figure 2.4 Living rosette leaf numbers in different treatments of Col-0 and *NahG* lines from3 wpi-8 wpi **A)** Average number of rosette leaves from 3wi to 8 wpi in Col-0 plants exposed to different treatments **B)** Average number of rosette leaves from 3wi to 8 wpi in *NahG* plants exposed to different treatments. (Legend: Green bars-Non-infested, Blue bars- infested with negative BCs, orange bars-infested with CLps positive BCs and CLps negative and brown bars-infested with CLps positive BCs and CLps positive). Data represents least square mean of rosette leaf numbers and standard error bars. A statistically significant difference ($p<0.05$, Repeated measures ANOVA) was detected in the number of rosette leaves between infested and non-infested plants for *NahG* at 7wpi. For both lines, CLps-positive plants had significantly ($p<0.05$, Repeated measures ANOVA) lower number of the rosette leaves than the CLps-negative at 8wpi

The number of internodes and internode lengths were also analyzed to determine the effect of treatments on stem growth. No significant differences were found in the number of internodes for both lines in response to CLps infection or psyllid feeding. However, at 8 wpi CLps positive plants had significantly (Fig 2.6, $p<0.05$, one-way ANOVA) shorter internodes in comparison to non-infected plants (non-infested, infested with negative BCs, Infested with positive BCs but CLps negative) confirming that stem development was affected by CLps infection. A very notable and distinct symptom of infected plants was an excessive growth of small size axillary leaves and clusters of cauline leaves for both lines (Fig 2.7). At 8 wpi, the number of axillary leaves and cauline leaves was significantly higher (Fig 2.8, $p<0.05$, one-way ANOVA) in infected plants compared to non-infected plants in both Col-0 and *NahG* plants.

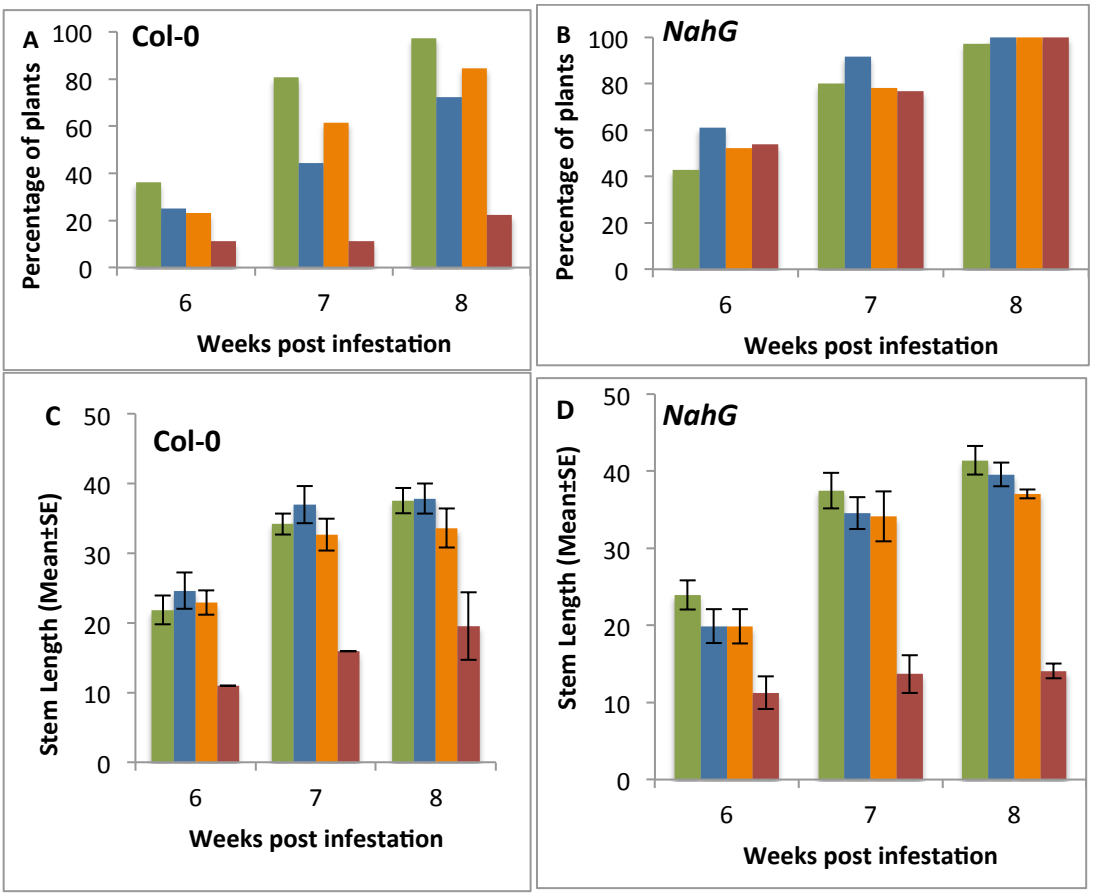

Figure 2.5: Effect of CLps infection on stem development and growth in Col-0 and *NahG* lines. **A)** Percentage of plants with stems from 6 wpi-8 wpi in Col-0 line **B)** Percentage of plants with stems from 6 wpi-8 wpi in *NahG* line **C)** Average stem length from 6 wpi to 8 wpi in Col-0, **D)** Average stem length from 6 wpi-8 wpi in *NahG.* (Legend: Green bars-Non-infested, Blue bars-infested with negative BCs, orange bars- infested with CLps positive BCs and CLps negative and brown bars – infested with CLps positive BCs and CLps positive).
For Col-0 line, the number of CLps-positive plants with stems was significantly lower (Chi-Square test, $p<0.05$) compared to the number of CLps-negative plants with stems. No significant difference was detected for *NahG* line in the number of CLps-positive with stems and CLps-negative plants with stems. Data represented in the graphs(C and D) is average stem length with standard errors. For both lines, CLps-positive plants had significantly shorter stems in comparison to CLps-negative plants from 6 wpi- 8 wpi ($p<0.05$, Repeated measures ANOVA)

Discoloration or purpling of leaves was observed on CLps positive plants around 5 wpi that became very distinct by 6-7 wpi. By 7 wpi some purpling was also evident in all treatments, but was significantly higher in CLps positive plants in comparison to CLps negative plants (Fig 2.9). In Col-0, 100% of CLps positive plants were discolored whereas in *NahG,* 84% of CLps positive plants were discolored. Another interesting symptom observed on the infected *Arabidopsis* plants was leaf curling. Since 6 wpi, leaf curling was consistently observed in almost all CLps positive plants for both Col-0 and *NahG* lines (Fig 2.10). The percentage of CLps positive plants with curled leaves was significantly higher than in that in CLps negative plants in both Col-0 and *NahG* lines. At 8 wpi, 100% of CLps positive plants had leaves curled irrespective of genetic background.

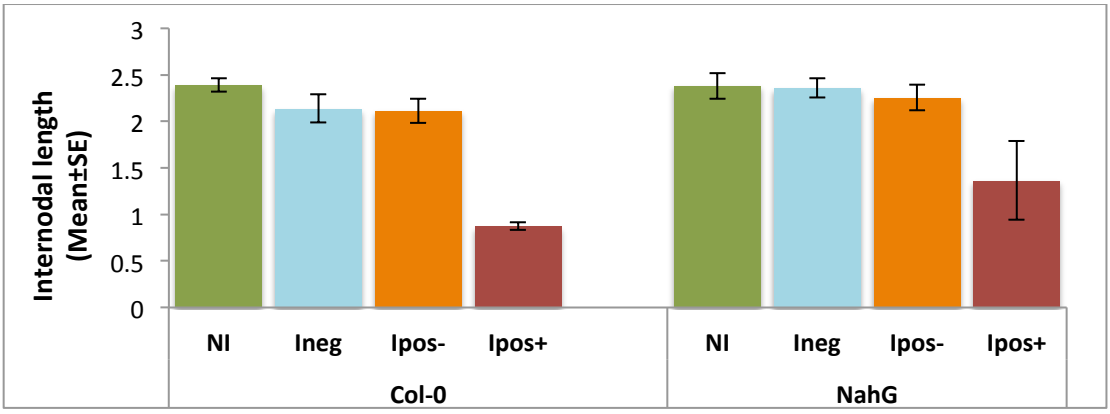

Figure 2.6: Average Internode length of Col-0 and *NahG* lines at 8 wpi**.** Left- Average internode length of Col-0 plants subjected to different treatments, Right- Average internode length of *NahG* plants subjected to different treatments. Data represented is the mean internode length with standard error bars for all replicates of the respective treatments. For both lines, the CLps-positive plants had significantly shorter internodes compared to CLps-negative plants ($p<0.05$, one-way ANOVA). (Legend: Green bars-Non-infested, Blue bars- infested with negative BCs, orange bars- infested with CLps positive BCs and CLps negative and brown bars – infested with CLps positive BCs and CLps positive)

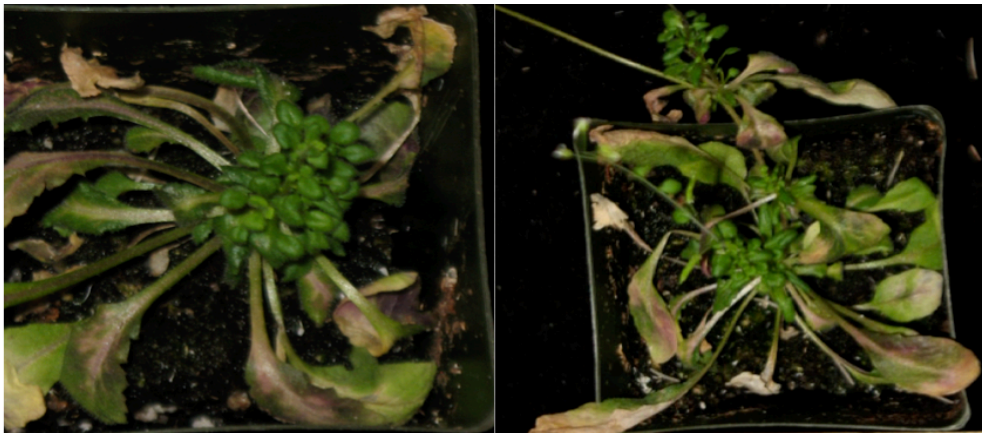

Figure 2.7: Excessive growth of axillary leaves (left) and clustered cauline leaves (right) of CLps infected plants.

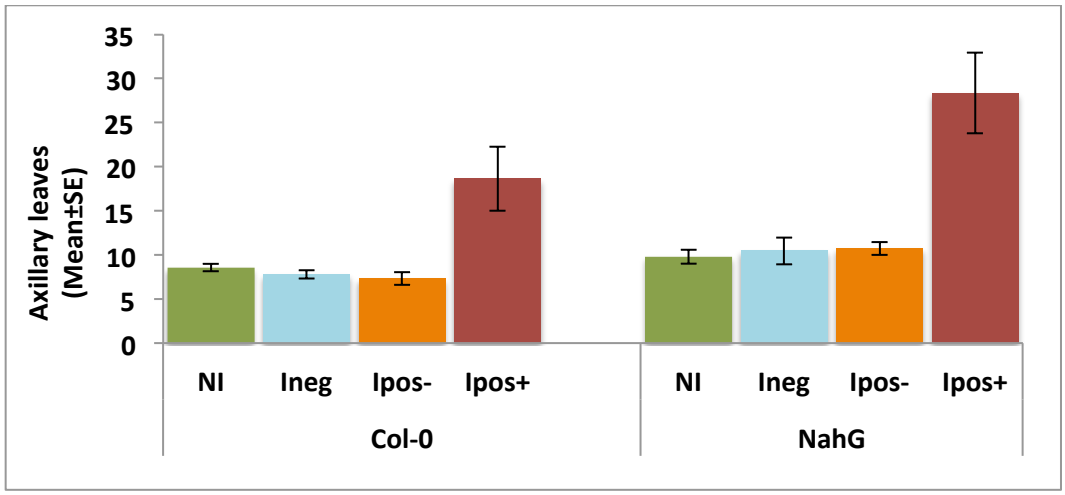

Figure 2.8: Average of axillary/cauline leaf numbers of Col-0 and *NahG* lines at 8 wpi. Left- Average length axillary leaves of Col-0 plants subjected to different treatments, Right- Average axillary leaves of *NahG* plants subjected to different treatments (Legend: Green bars-Non-infested, Blue bars- infested with negative BCs, orange bars- infested with CLps positive BCs and CLps negative and brown bars – infested with CLps positive BCs and CLps positive). The results shown are the means of the number of axillary leaves for all replicates in the respective treatments with standard error bars. For both lines, the number of axillary/cauline leaves was significantly higher in CLps-positive plants compared to CLps-negative plants ($p<0.05$, one-way ANOVA) at 8wpi.

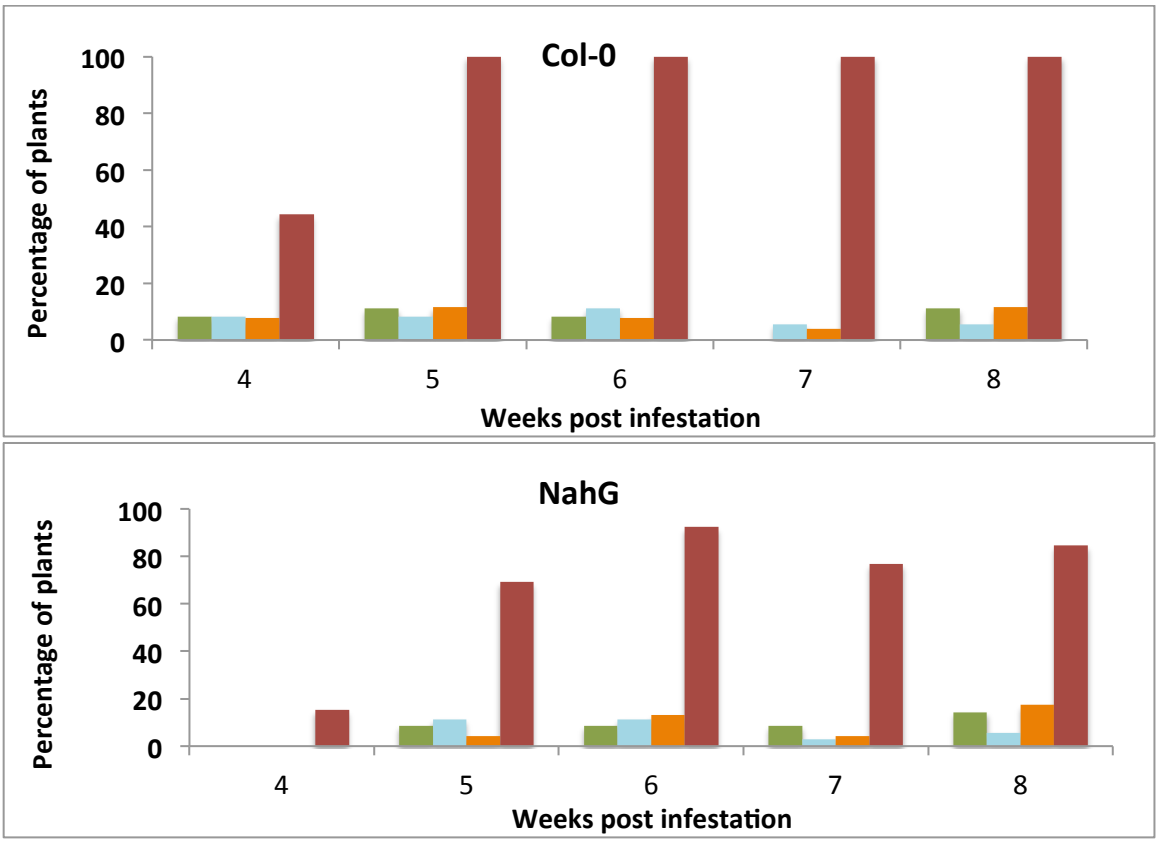

Figure 2.9A-B: Discoloration / purpling of plants in different treatments of Col-0 and *NahG* lines. A) Percentage of discolored plants in different treatments of Col-0 line B) Percentage of discolored plants in different treatments of *NahG* line. The number of infected (CLps-positive) discolored plants was significantly higher (Chi-square test, $p<0.05$) than non-infected (CLps-negative) discolored plants for both Col-0 and *NahG* lines from 5 wpi to 8 wpi

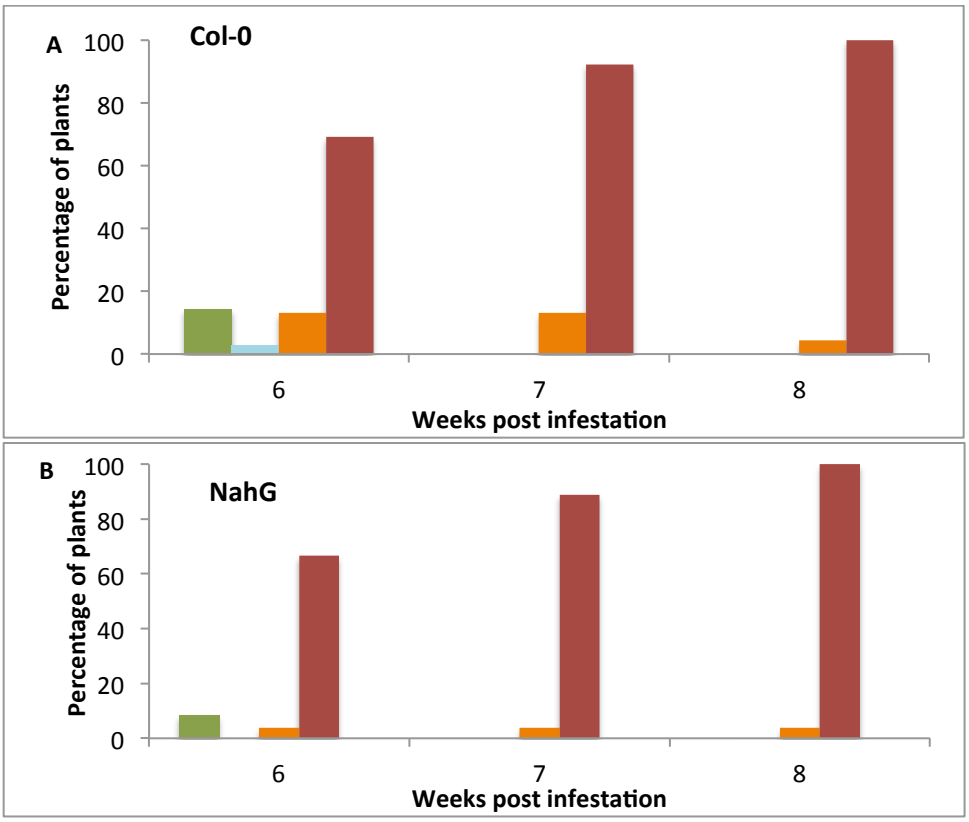

Figure 2.10 A-B: Percentage of plants with curled leaves in different treatments of Col-0 and *NahG* lines. A) Percentage of Col-0 plants in different treatments with curled leaves B) Percentage of *NahG* plants in different treatments with curled leaves. The number of infected (CLps-positive) plants with curled leaves was signifcantly higher (Chi-square test, $p<0.05$) than non-infected (CLps-negative) with curled leaves plants for both Col-0 and *NahG* lines from 6 wpi to 8 wpi

Preliminary processing and quality assessment of microarray data

The consistency in different parameters such as background values, internal control genes was assessed for determining the labeling and hybridization quality of the microarray. Raw and log transformed intensity images for all chips were analyzed and no regional bias in labeling was detected. Within each treatment, the background value for all replicate chips was consistent. Background values ranged from 34.2-27.9 and the

mean value was 30.15 with standard deviation of 1.77 (Table 2.2). The scaling factor was less than 3.00 and was similar in all replicates (difference of ±0.5max) within treatment and across lines (Table 2.2). The internal control genes also showed consistent expression across all replicates and treatments.

Table 2.2. Quality assessment of different replicates and different treatments of *NahG* and Col-0 lines.

QA Metrics	Background	Raw Q	Scaling factor	Percent present calls	GADPH 3' to 5' ratio	β -actin 3' to 5' ratio
Non-infested *NahG*						
POOL1 (BLOCK1)	34.2	1.2	2	59.6%	1.3	2.0
POOL2 (BLOCK2)	33.4	1.2	2.1	57.6%	1.2	1.9
POOL3 (BLOCK3)	32.5	1.1	2.5	54.9%	1.2	1.9
Non-infested Col-0						
POOL4 (BLOCK1)	33.2	1.1	2.5	57.9%	1.2	1.9
POOL5 (BLOCK2)	31.6	1.1	2	58.9%	1.2	1.9
POOL6 (BLOCK3)	31.8	1.0	2	58.3%	1.2	1.5
Infested with CLps-negative psyllids *NahG*						
POOL7 (BLOCK1)	29.3	0.9	2.8	57.2%	1.8	2.0
POOL8 (BLOCK2)	30.8	1.0	2.8	56.6%	1.2	`
POOL9 (BLOCK3)	29.9	1.0	3	51.7%	1.8	2.0
Infested with CLps-negative psyllids Col-0						
POOL10 (BLOCK1)	28.6	1.0	3	54.0%	1.5	2.0
POOL11 (BLOCK2)	29.7	1.0	2.8	54.4%	1.5	1.8
POOL12 (BLOCK3)	30.2	1.0	2.8	54.8%	1.5	1.8
Infested with CLps-positive psyllids *NahG* (Symptomatic)						
POOL13 (BLOCK1)	29.4	1.0	2.9	57.8%	1.9	1.9
POOL14 (BLOCK2)	29.6	1.0	3	53.7%	1.5	2.0
POOL15 (BLOCK3)	31.3	1.0	2.9	55.2%	1.5	2.1
Infested with CLps-positive psyllids Col-0 (Symptomatic)						
POOL16 (BLOCK1)	29.2	1.0	2.2	58.8%	1.2	1.8
POOL17 (BLOCK2)	28.4	1.0	2.2	58.6%	1.3	2.0
POOL18 (BLOCK3)	29.0	1.0	2	60.9%	1.0	2.0
Infested with CLps-positive psyllids *NahG* (Asymptomatic)						
POOL19 (BLOCK1)	27.9	0.9	2.1	60.1%	1.0	2.0
POOL20 (BLOCK2)	28.8	1.0	2	59.5%	2.0	2.2
POOL21 (BLOCK3)	28.4	0.9	2	60.3%	1.0	1.8
Infested with CLps-positive psyllids Col-0 (Asymptomatic)						
POOL22 (BLOCK1)	29.2	0.9	2	61.0%	1.0	1.8
POOL23 (BLOCK2)	28.5	0.9	3	52.1%	1.8	2.0
POOL24 (BLOCK3)	28.9	0.9	2.2	57.3%	1.2	2.0

Each pool represents RNA pooled from three plants with same treatment and hybridized to single microarray.

Microarray data analysis revealed many differentially expressed transcripts in *Arabidopsis thaliana* in response to *Candidatus* Liberibacter psyllaurous infection.

To understand *Arabidopsis* responses to CLps infection, transcriptional changes in *NahG* and Col-0 lines were analyzed for four treatments. The four treatments were A) plants infested with CLps negative BCs B) plants infested with CLps positive BCs, positive for CLps and symptomatic C) plants infested with psyllids with CLps positive BCs, negative for CLps and asymptomatic and D) non-infested plants (Fig 2.1). For each line, RNA samples from three plants of each treatment were pooled together for microarray hybridization. Among 22,500 probe sets of ATH1 gene chip, the number and percent present calls were 12375 (55%) and 13500 (60%) were detected in symptomatic *NahG* and Col-0 plants positive for CLps infection at 8 wpi. Of the probe sets called present, 130 and 52 were significantly ($p<0.05$) differentially expressed transcripts in response to CLps infection in Col-0 and *NahG* lines (Table 2.3) respectively. These transcripts were identified by comparing responses in plants infested with CLps positive BCs and positive for CLps with plants infested with CLps negative BCs and negative for CLps for both lines. For both lines, the number of up-regulated genes was higher than the number of down-regulated genes. Comparative analysis of DEGs in CLps positive plants revealed that few genes were expressed in both lines and many genes showed line specific expression. Among the total DEGs in *NahG* and Col-0, 22 up-regulated genes and 9 down-regulated genes were expressed in both lines (Fig 2.11). Interestingly, there were several (72 up-

regulated and 29 down-regulated) genes that were expressed only in Col-0 and not in *NahG* whereas there were few (19 up-regulated and 8 down-regulated) genes that were expressed in only *NahG* and not in Col-0 (Fig 2.11). At a cut off of fold change 4, the number of down-regulated genes was very low (5 DEGs for of *NahG* and 3 for Col-0) and hence the cutoff was adjusted to 2 to include more significantly down-regulated transcripts. All the differentially expressed genes (at significance level $p \leq 0.05$, fold change ≥ 4 for up-regulated, $p \leq 0.05$, fold change ≥ 2 for down-regulated) were grouped into different gene ontology categories of biological function and cellular components (Fig 2.12 and Fig 2.13). At $p \leq 0.05$, no significant differentially expressed genes were identified in the comparative analysis of non-infested plants and the plants infested with CLps negative psyllids for both lines (Table 2.3) The number of DEGs identified from comparison of CLps positive plants to two treatments A) plants with CLps negative BCs and detected negative for CLps and B) plants with CLps positive BCs and detected negative for CLps were similar for both lines. For functional categorization of all DEGs in response to CLps infection, I have used DEGs identified from the comparison of CLps positive plants with plants infested with CLps negative BCs and detected negative for CLps.

Table 2.3: Number of differentially expressed genes in different treatments of Col-0 and *NahG* lines

Lines	Treatments Comparison	Number of DEGs*	Up- regulated Fold change ≥ 4	Down regulated Fold change ≥ 2
Col-0	INeg_NI	0	0	0
	IPos-ve_NI	0	0	0
	IPos+ve_NI	122	76	46
	IPos+ve_IPos-ve	114	73	41
	IPos+ve_INeg	130	92	38
	IPos-ve_INeg	0	0	0
NahG	INeg_NI	0	0	0
	IPos-ve_NI	0	0	0
	IPos+ve_NI	142	54	88
	IPos+ve _IPos-ve	54	33	21
	IPos+ve_INeg	52	36	16
	IPos-ve_INeg	0	0	0

*DEGs- Differentially expressed genes

Note: NI: Non-infested, INeg: Infested with negative BCs, IPos-ve: infested with CLps positive BCs and CLps negative, IPos+ve: Infested with CLps positive BCs and CLps positive

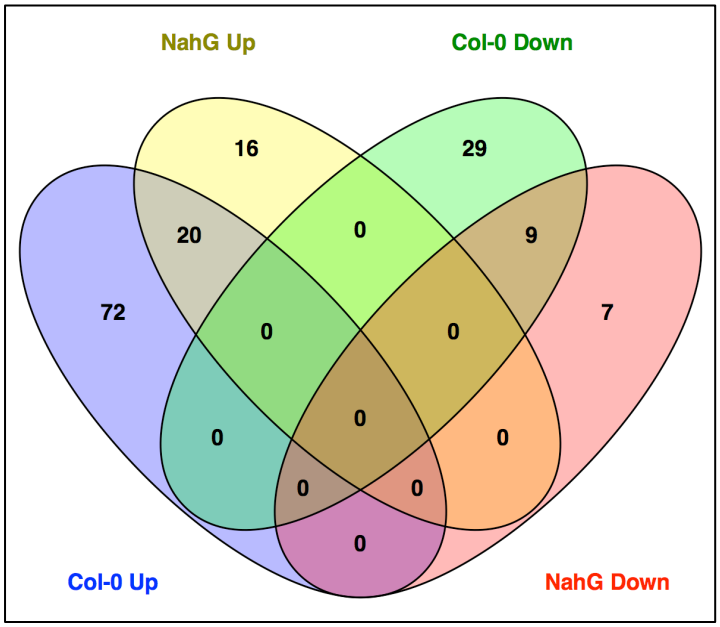

Figure 2.11: Venn diagram of number of differentially expressed transcripts in response to *Candidatus* Liberibacter psyllaurous infection. DEGs in plants Infested with CLps positive BCs, and detected CLps positive versus Infested with CLps negative BCs, and detected CLps negative plants of NahG and Col-0. (Up- Up regulated genes Fold change ≥ 4, down- down regulated genes Fold change ≤ 2)

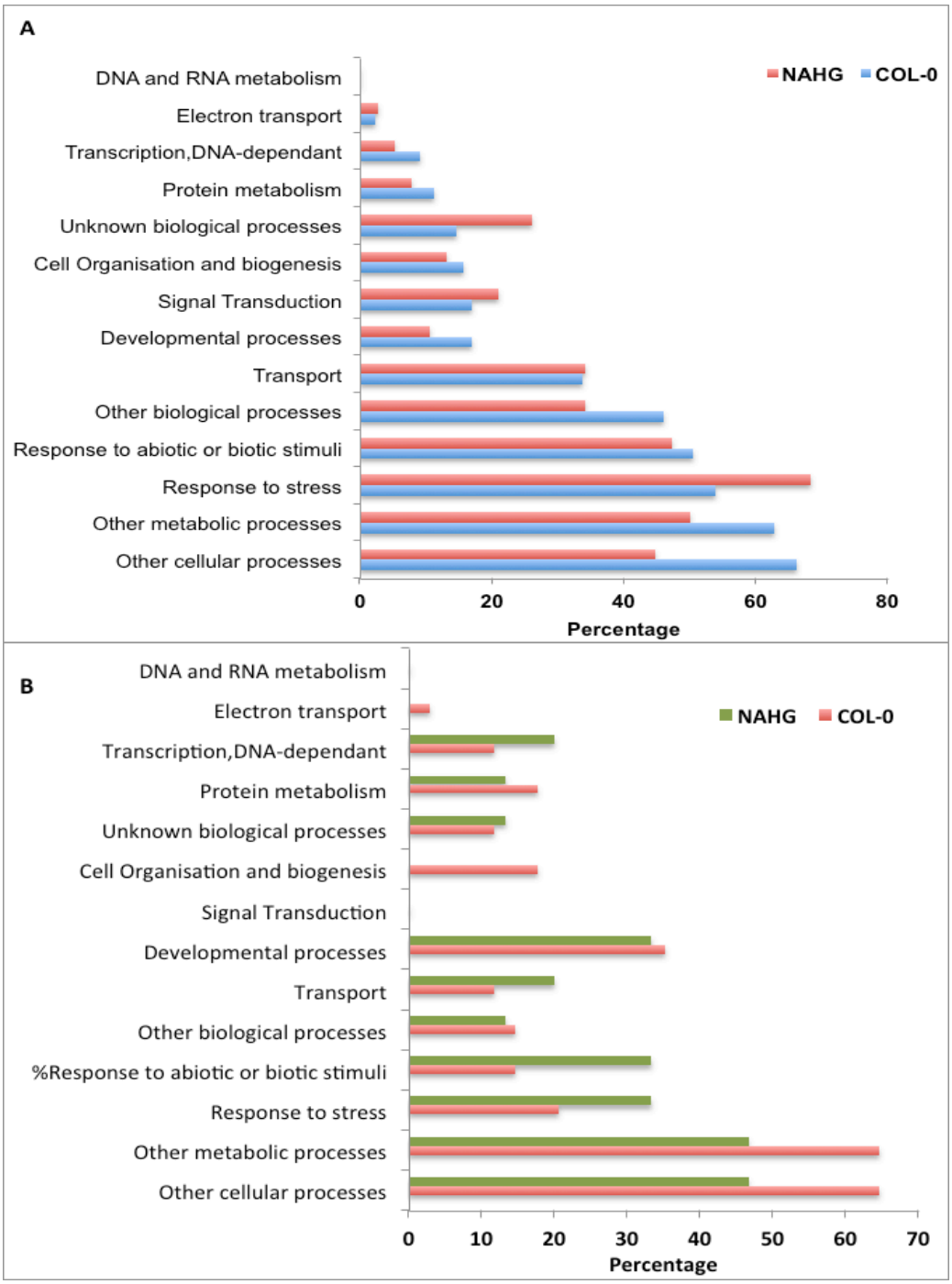

Figure 2.12: Functional categorization by Gene Ontology categories of biological function of genes differentially expressed in response to CLps infection **A:** Top-Functional categorization of genes up-regulated in *NahG* and Col-0.**B:** Bottom-Functional categorization of genes down-regulated in NahG and Col-0

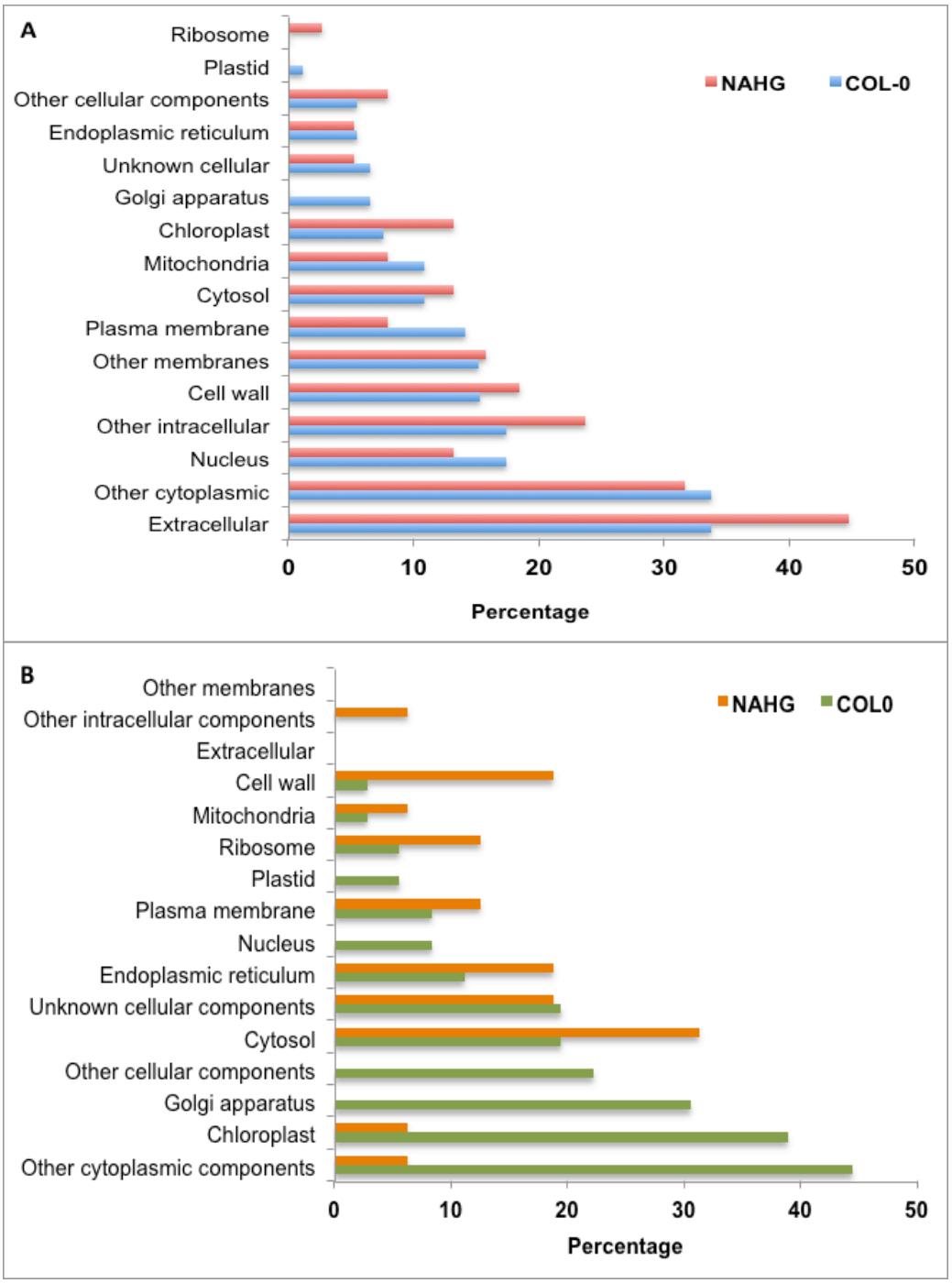

Figure 2.13: Functional categorization by Gene Ontology categories of cellular components of genes differentially expressed genes in response to CLps infection. **A:** Top-Functional categorization of genes up-regulated in *NahG* and Col-0. **B:** Bottom-Functional categorization of genes down-regulated in *NahG* and Col-0.

Functional categorization of differentially regulated genes in response to *Candidatus* Liberibacter psyllaurous infection

In response to CLps infection, the majority of differentially up-regulated and down-regulated genes belonged to the biological processes categories of response to abiotic or biotic stimuli, response to stress and other cellular and metabolic processes (Fig 2.12A, B). In addition to these categories, about several genes in developmental processes were significantly down-regulated for both lines (Fig 2.12B). Of the total up-regulated genes, 45% and 32% encode proteins that are located extracellular in *NahG* and Col-0 respectively. It was interesting to observe the differences in cellular components categorization of down-regulated genes for both lines. In *NahG*, 50% of down-regulated genes encode proteins located in cell wall and cytosol (Fig 2.13B) and in Col-0 line, majority of down-regulated genes (75%) encode products that were located in Golgi apparatus and chloroplast (Fig 2.13B). In *NahG*, no down-regulated gene was associated with Golgi apparatus and less than 10% of genes were associated with chloroplast. Based on the gene ontology categorization of DEGs in biological processes, it is evident that *Arabidopsis* triggers defense responses to CLps infection. The following sections describe in detail the differentially expressed genes in *NahG* and Col-0.

Response to abiotic or biotic stimuli and stress category:

In both Col-0 and *NahG* lines, plants detected positive for CLps infection were symptomatic at 8 wpi and showed significant differential regulation of several defense genes that belong to this category (Table S2.2, S2.3). Of the total of 130 and 52 DEGs in the infected Col-0 and *NahG* plants, 39 and 23 up-regulated genes belonged to this category in Col-0 and *NahG* respectively. Among these, genes up-regulated significantly in Col-0 but not in *NahG* include several transcription factors such as *WRKY 38*, *WRKY 70*, *WRKY 54, WRKY75*, *LATE UP-REGULATED IN RESPONSE TO HYALOPERONOSPORA PARASITICA* (*LURP1*), *PATHOGENESIS RELATED PROTEIN-1* (*PR1*), *PATHOGEN AND CIRCADIAN CONTROLLED 1* (*PCC1*), and *GLUTAREDOXIN* family protein. Transcription factor WRKY38 interacts with HISTONE DEACETYLASE19 along with WRKY62 and is involved in triggering of basal defense responses in plants (Kim et al., 2008). WRKY70 is known to be activated by SA and repressed by JA, and is critical in modulation of plant responses against bacterial and fungal pathogens that typically use SA and JA defense pathways respectively (Li et al., 2004). The *PCC1* transcript was induced eleven fold more in CLps infected compared to non-infected Col-0 plants. PCC1 is a trans-membrane protein and as the name indicates, it is regulated by salicylic acid and expresses differently based on the plant's circadian clock. In *NahG* and Col-0, transcripts of *OSMOTIN34* (*AtOSM34*), *KUNITZ TRYPSIN INHIBITOR* (*AtKTI*, AT1G73260), *PATHOGENESIS RELATED PROTEIN 2* (*PR2*), *PATHOGENESIS RELATED PROTEIN 5* (*PR5,* Fig

2.14), and *BASIC CHITINASE* (*PR3*) showed similar fold change in response to pathogen infection. *AtOSM34* and *AtKTI1* modulate cell signaling and programmed cell death in response to different pathogens respectively (Abdin et al., 2011; Li et al., 2008). These genes showed 50.43 and 69.43 fold change in Col-0 and 6.4 and 11.43 fold change in *NahG* respectively in response to CLps infection (Table S2.2, S2.3). The β-*1-3 GLUCANASE 2* (also known as *PR2)* was up-regulated by 30 and 47 fold in Col-0 and *NahG* lines respectively. Plant natriuretic peptide A, a long distance signaling molecule functioning via phloem in systemic acquired resistance and plant homeostasis, was up-regulated by 24 fold and 13 fold in *NahG* and Col-0 respectively.

Other cellular processes and metabolic processes

The next major category of the genes that were induced and repressed was cellular and metabolic processes (Table S2.5, S2.6). In this category, three genes *METHIONINE SULFOXIDE REDUCTASE B7* (*MSRB7*), *METHIONINE SULFOXIDE REDUCTASE B8* (*MSRB8*), and *GLUTAMATE DEHYDROGENASE 2* (*GDH2*) were up-regulated more than 4 fold in both Col-0 and *NahG* lines. The *GDH2* gene is expressed in mitochondria in response to alteration in redox conditions of the cell (Tarasenko et al., 2009). Methionine sulfoxide reductase genes are expressed in the chloroplast and cytosol. Proteins with higher Met residues are more susceptible to oxidation by reactive oxygen species and can become non-functional. Methionine sulfoxide reductase restores such oxidized proteins by reduction and renders them functional in processes (Lee et al., 2014). In *NahG*, genes

nicotianamine synthase1, which is involved in Fe mediated homeostasis (Klatte et al., 2009) and Glutamate decarboxylase which is involved in nitrate assimilation and transport (functional annotation from TAIR) were induced by 4.8 and 7.8 fold respectively in response to infection. Several genes encoding enzymes such as GLYCOSYL HYDROLASE SUPERFAMILY PROTEIN, β-XYLOSIDASE 1, LACTOYLGLUTATHIONE LYASE / GLYOXALASE I FAMILY PROTEIN, ALPHA-AMYLASE-LIKE, β-GALACTOSIDASE 4 and β-GLUCOSIDASE 30 that are involved in carbohydrate metabolism were induced more than 10 fold in Col-0. Among the down-regulated genes in this category, the only gene differentially expressed in both Col-0 and *NahG* encodes THIAMIN DIPHOSPHATE-BINDING FOLD SUPERFAMILY PROTEIN (THDP-binding, AT5G09300), which is involved in oxidoreductase activity (functional annotation from TAIR). In *NahG,* the most repressed gene (2 fold change) in this category encodes a SUCROSE-PHOSPHATE SYNTHASE FAMILY protein that is involved in sucrose biosynthesis whereas in Col-0 the most repressed gene (fold change of 3.5) was a 60S acidic ribosomal protein family gene that specifies a ribosomal protein. In Col-0, a majority of down-regulated genes encode proteins located in the chloroplast. These genes were *CYTOCHROME P450 FAMILY 97, SUBFAMILY A, POLYPEPTIDE 3, ASPARTATE-GLUTAMATE RACEMASE FAMILY PROTEIN*, *HALOACID DEHALOGENASE-LIKE HYDROLASE FAMILY PROTEIN*, *PROTEIN PHOSPHATASE 2C FAMILY PROTEIN, PHOSPHOGLYCERATE MUTASE FAMILY PROTEIN, DAD1-LIKE LIPASE 2* and *NAD(P)-LINKED OXIDOREDUCTASE SUPERFAMILY PROTEIN*. Most of these genes encode enzymes involved in oxidation-reduction processes.

Transport:

Among the DEGs in this category, only one gene encoding, bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein (AT5G05960), was induced in both Col-0 and *NahG* with same fold change. This gene is involved in lipid transport. Genes involved in transport such as CASP like protein 1 D1 involved in amino acid and nitrate transport, heavy metal transport/detoxification superfamily protein (HIPP) also known as metallochaperone involved in transporting metal ions inside the cell, vacuolar sorting receptor 7 involved in nitrate transport, were induced only in Col-0 in response to CLps infection. Peptide transporter 3 involved in transporting dipeptide or tripeptides was also induced only in Col-0. In *NahG*, genes encoding proteins involved in sodium / cation transport (CHX20) and proline transport were significantly down-regulated. In Col-0, genes involved in amino acid transport and potassium transport activity was down-regulated by 2 and 3 fold respectively.

Developmental processes

Among up-regulated genes, no gene was classified under the developmental process category in the *NahG* line, however several genes involved in developmental processes were up-regulated in Col-0. Among these genes were NAC domain containing protein 47 and NAC domain containing proteins that exhibit transcription factor activity in meristem development. Genes *SENESCENCE RELATED GENE 1* (*SRG1*) and *SAUR-LIKE AUXIN-RESPONSIVE PROTEIN* that are involved in involved in plant senescence were up-

regulated more than 16 fold and 8 fold in response to infection respectively. Among down-regulated genes, about 35% were catergorized with developmental processes for both lines. Interestingly, genes encoding transcription factors AGAMOUS LIKE 42, AGAMOUS LIKE 3 and AGAMOUS LIKE 8 involved in floral development were repressed in both *NahG* and Col-0 lines. Their lower expression could be a possible reason for the slower development of floral organs in CLps infected plants. In Col-0, the two notable down-regulated genes (repressed more than 8 and 4 fold) encode GLUTAREDOXIN and THIOREDOXIN that regulate cell redox homoeostasis.

Unknown Biological processes

This category included all the differentially expressed genes not classified in any other categories. Notable genes in this category were lectins such as *JACALIN RELATED LECTIN PROTEIN 22* and other mannose binding lectins (AT1G78860, AT1G78850). The JACALIN RELATED LECTIN 22 protein is reported to bind to beta glucosidase and regulate its polymerization in formation of ER bodies during a defense response (Nagano et al., 2008). In Col-0, the down-regulated genes encode methyl esterase (more than 8 fold change), plant invertase (more than 2 fold change), and enzymes involved in hydrolase and enzyme inhibitor activity. A notable gene that was down-regulated by 10 fold in *NahG* encodes PYRIDOXAL PHOSPHATE (PLP)-DEPENDENT TRANSFERASES superfamily protein.

Validation of microarray results of 13 candidate genes at 3 weeks post infestation and 8 weeks post infestation by quantitative real time PCR

To validate the expression of the candidate genes, an independent experiment was conducted with plants infested with CLps negative psyllids and CLps positive plants. Same-stage leaves were collected from plants from both treatments at 3 weeks post infestation and 8 weeks post infestation. All samples were tested for CLps infection at 8 wpi to select CLps positive and negative plants. DNAase treated RNA samples from three biological replicates for each treatment were used for real time quantitative PCR to determine fold change between CLps positive and CLps negative plants. Fifteen candidate genes were selected and their fold change was analyzed at 3 wpi and 8 wpi. Of the 15 selected genes, 13 genes showed similar up-regulation or down-regulation by quantitative real time PCR as microarray results at 8 wpi (Fig 2.14). The qRT-PCR expression analysis of candidate genes at 3 wpi indicates that not all genes were induced at this time point but most were induced significantly by 8 wpi. At 3 wpi, only *PR5, OSM34, BGLU30* transcripts showed similar fold change to that of 8 wpi in both lines. Analysis of the fold change difference by a different set of replicates confirmed the differential transcript levels from microarray experiment.

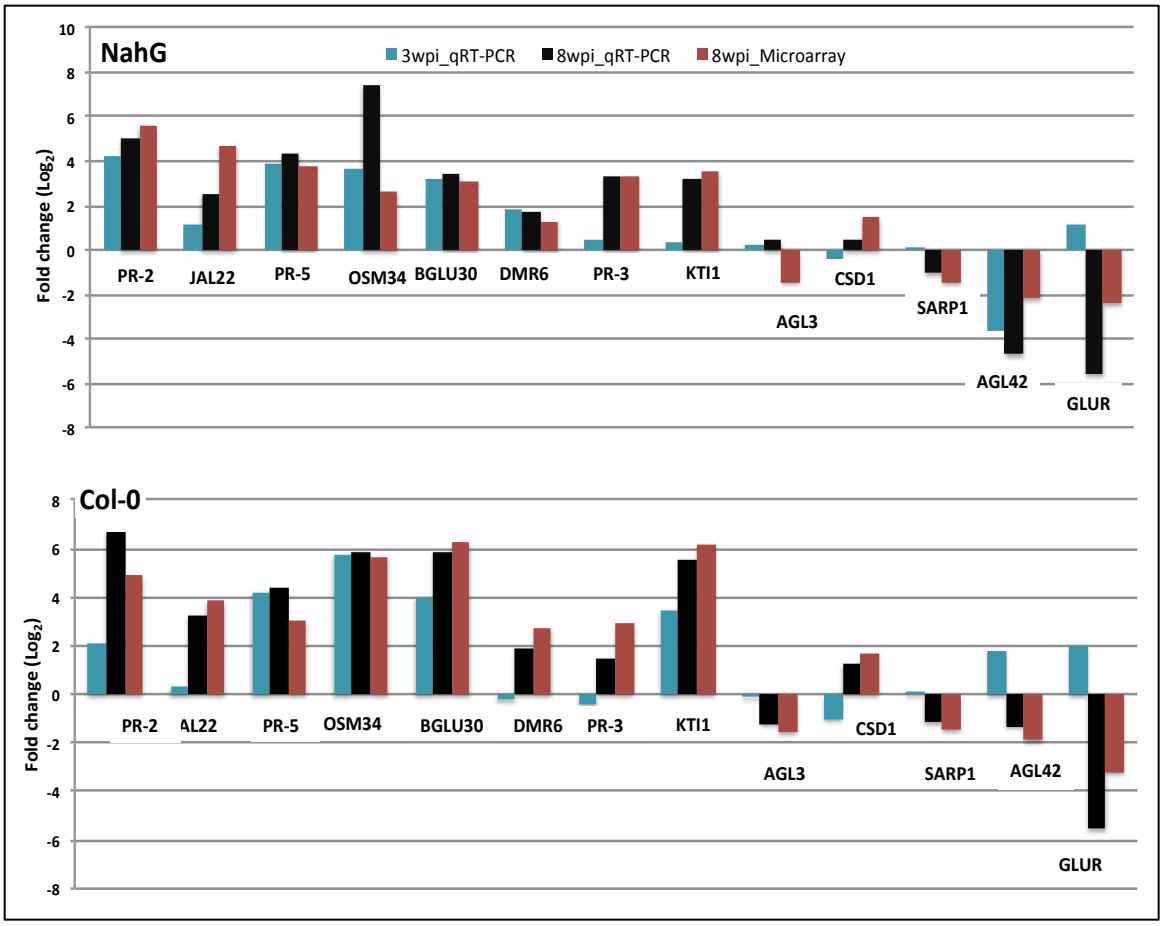

Figure 2.14: qRT-PCR validation of differentially expressed genes in response to CLps infection. Top: Fold Change (Log_2) in transcript levels of candidate genes in *NahG*. Bottom: Fold change in transcript levels (Log_2) of candidate genes in Col-0.
Legend: 3 wpi-qRT-PCR, Blue bars: transcript levels detected by qRT-PCR at 3 wpi,
8 wpi-qRT-PCR, Black bars: transcript levels detected by qRT-PCR at 8 wpi,
8 wpi-Microarray, red bars: transcript levels detected by microarray.

DISCUSSION

To decipher interactions between a plant and pathogen, it is crucial to understand the plant's tolerance or susceptibility to the pathogen based on not only the alterations in plant development or phenotype but also on understanding the molecular mechanisms at the gene or protein levels. In this study, we attempt to understand *Arabidopsis thaliana* responses to *Candidatus* Liberibacter psyllaurous pathogen at the phenotypic and genetic levels. The detection of high numbers of CLps positive plants having bacterial cells in young, new leaves and stems indicate that *Candidatus* Liberibacter psyllaurous spreads systemically in the plants. The first symptoms to be observed on the infected plants were curling of new leaves at the rosette center and purpling of leaf veins, which became distinctly visible by 6 wpi. By 8 wpi almost all infected plants had intense purple coloration. Frequency analysis of plants with purple coloration showed that a significant percentage of CLps infected plants had discolored or purpling of leaves in comparison to non-infested or non-infected plants. Leaf and stem growth were analyzed in various treatments and significant differences in number of rosette leaves, stem development and length, and number of axillary leaves were found at 8 wpi. These differences indicate that growth of CLps-infected plants was significantly slower compared to non-infected plants for both the Col-0 and *NahG* lines. CLps infection cause significant deterioration in growth in Col-0, which was evident in absence of stems, fewer leaves, and distinct discoloration of leaves. In the *NahG* line,

CLps infected plants developed stems, flowers and siliques indicating growth was less affected by infection than in Col-0. Symptoms on plants ranged from severe to moderately severe on infected plants but this severity did not correspond to the number of bacterial cells especially with the *NahG* line. To understand changes in gene expression patterns for *NahG* and Col-0 lines, we investigated induction and suppression of transcripts using a microarray for four treatments at 8 wpi: 1) non-infested plants, 2) plants infested with CLps negative BCs, 3) plants infested with CLps positive BCs, positive for CLps and symptomatic and 4) plants infested with CLps positive BCs, negative for CLps and asymptomatic. No significant differences in gene expression were detected as the result of BC feeding on either *NahG* or Col-0 lines. However, the changes in gene expression in response to psyllid feeding may occur because it seems likely that genes expressed early after infestation may return to normal expression levels by 8 weeks after infestation. Several genes showed significant differential expression in response to CLps infection in *NahG* and Col-0. The number of differentially expressed genes was more in Col-0 than *NahG*. Gene ontology enrichment analysis (Fig 2.15) of differentially expressed transcripts indicates that *Arabidopsis thaliana* triggers several responses that contribute to basal defense to CLps infection.

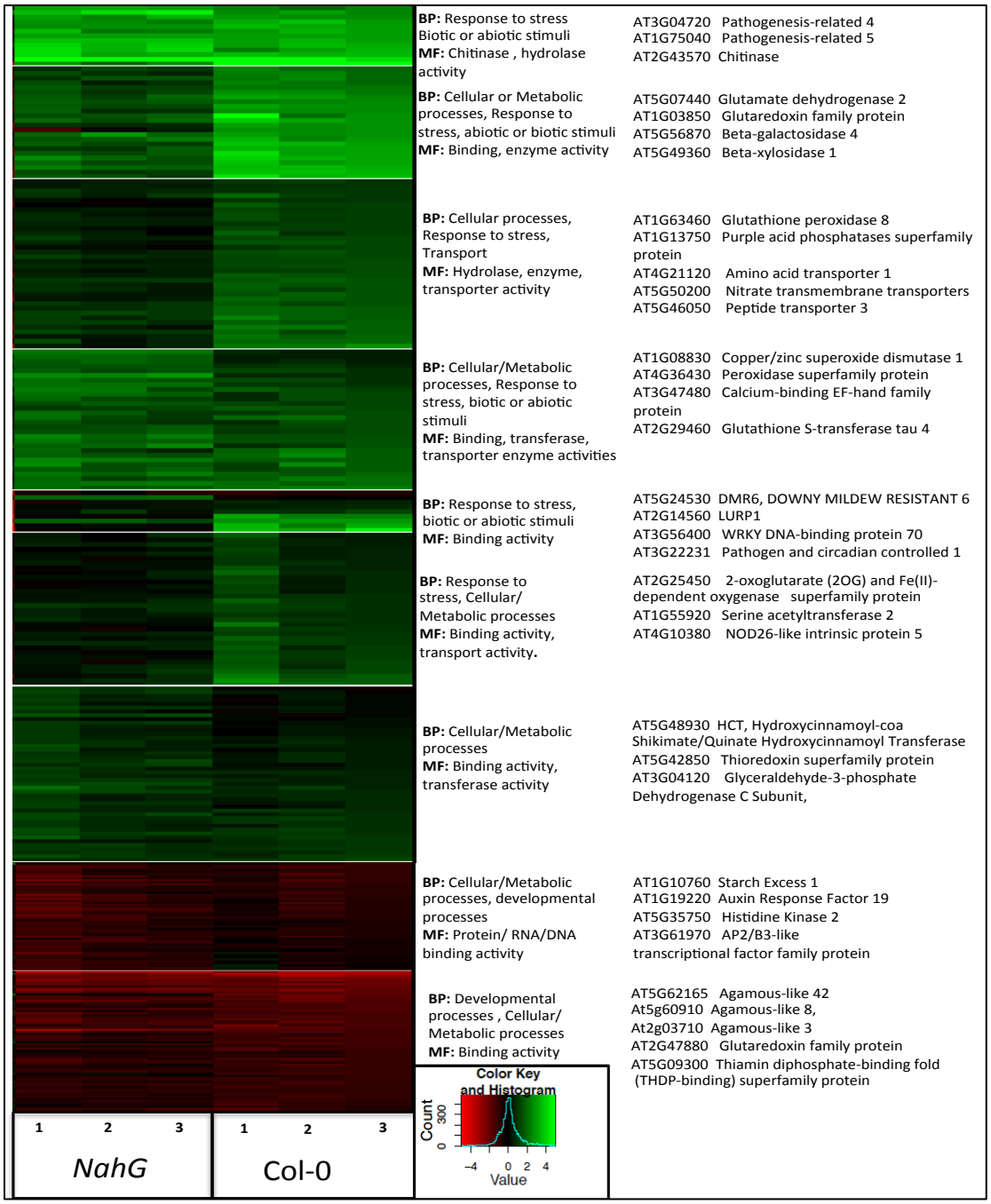

Figure 2.15: Gene ontology enrichment analysis of CLps infected plants of *NahG* and Col-0 lines compared with three classes of non-infected plants (1, 2,and 3). HeatMap shows nine clusters of genes with similar expression profile in CLps infected plants when compared to treatments 1) non-infested plants, 2) plants infested with CLps positive BCs and negative for CLps 3) plants infested with CLps negative BCs and negative for CLps. Selected genes are shown as representative for each cluster. **Legend/Abbreviations**: BP: Biological Processes, MF: Molecular function.

The most enriched GO terms of biological processes were response of biotic or abiotic stimuli or stress and cellular and metabolic processes (Fig 2.15) for both lines. Induction of genes encoding enzymes such as peroxidase, copper superoxide dismutase, Glutathione S-transferase that react to reactive oxygen species (ROS) are an indication of PAMP (Pathogen associated molecular patterns) triggered defense responses. Elicitation of hypersensitive response in form of oxidative burst is a usually first form of plant defense signaling mechanism to restrict bacterial growth (Apel and Hirt, 2004; Baxter et al., 2013). However, the regulation of ROS by superoxide dismutase and other enzymes is crucial to avoid excessive cell death during stress (Apel and Hirt, 2004). In response to CLps infection, up-regulation of genes encoding calcium binding EF protein (AT3G47480) and DMR6 (2-oxoglutarate Fe(II) dependent oxygenases) is an evidence of plant's attempt to regulate the innate immune responses. Similar to HLB disease related gene expression (Fan et al., 2012), many pathogenesis-related proteins and WRKY transcription factors were significantly up-regulated in Col-0. Pathogenesis-related proteins involved in systemic acquired resistance were chitinase enzymes (PR4 and PR3), β-1,3-GLUCANASE and PR5 (THAUMATIN). These genes showed similar expression changes in the *NahG* and Col-0 lines with the exception of the *PR 1* gene, which was significantly expressed only in Col-0. β-1,3-GLUCANASE, an enzyme involved in degradation of callose and thereby regulating its deposition (Beffa et al., 1996) was expressed at 29 fold and 47 fold higher in infected than non-infected plants in Col-0 and

NahG line. These results could be an indication of callose deposition and its consequent degradation in *Arabidopsis* plants but this should be confirmed by anatomical and biochemical analysis of infected tissues. In HLB, most of the disease related symptoms are associated to phloem blockage by callose and phloem protein 2 (PP2), however in our studies no *Arabidopsis* ortholog of the *PP2* gene was detected in infected plants. In the *NahG* line, the salicylic acid pathway was suppressed and consequently several WRKY transcription factors involved in the downstream signaling pathway were not induced. Among the up-regulated WRKY transcription factors in Col-0 were WRKY 45 and WRKY 75 which are known to be induced in response to phosphate starvation (Devaiah et al., 2007; Wang et al., 2014). These results indicate that CLps infection may influence phosphate levels in *Arabidopsis*, a remarkable similarity to citrus/HLB responses (Zhao et al., 2013). Several peroxidase family proteins were up-regulated more than four-fold in Col-0 whereas only one gene was up-regulated more than four-fold and significantly in *NahG*. Peroxidase catalyzes oxidoreduction between hydrogen peroxide and reducing agents and is specifically induced in response to plant hormones including salicylic acid, jasmonic acid and abscisic acid. In addition to peroxidases, two defense responsive genes *OSMOTIN 34* and *KUNITZ TRYPSIN INHIBITOR* were notably expressed in *NahG*, Col-0 and in HLB infected citrus. The Pathogen and circadian controlled 1 gene (*PCC1*) was induced only in Col-0. It is regulated in a salicylic acid dependent manner by the circadian clock (Sauerbrunn and Schlaich, 2004). Lack of *PCC1* expression in the *NahG* line corresponds to zero or low levels SA in plants (Segarra et al.,

2010). The *CCA1* gene, which functions upstream of *PCC1* shows increased expression with lower SA levels (Segarra et al., 2010). In HLB infected citrus, *CCA1* is down-regulated, which could result from increased SA levels and would be expected to result in an increased expression of *PCC1* gene. Sulphur, nitrogen and carbon pathways are dependent on the circadian clock and alteration in these genes (*PCC1, CCA1*) can affect the downstream metabolic process (Harmer et al., 2000). Genes encoding enzymes **α**-AMYLASE-like, β-GALACTOSIDASE 4 and β-GLUCOSIDASE 30, involved in carbohydrate metabolic processes, were significantly up-regulated in CLps infected plants indicating alteration in starch metabolism similar to Huanglongbing disease. Several similarities in transcriptomic changes between Citrus HLB disease and *Arabidopsis*-CLps show similarity in the plant responses to *Candidatus* Liberibacter species. Most down-regulated genes encode proteins that were located in chloroplasts or were involved in developmental processes. Other notable down-regulated genes in Col-0 were *GLUTATHIONE-S-TRANSFERASE TAU5* and *AUXIN RESPONSE FACTOR 2.* Both GLUTATHIONE-S-TRANSFERASE TAU5 and AUXIN RESPONSE FACTOR 2 are induced by auxins (Van der Kop et al., 1996) and functions in auxin mediated signaling and their down regulation can affect plant senescence and floral transition (Ellis et al., 2005; Okushima et al., 2005). Down-regulation of several MADS box transcription factors *AGL42, AGL8, AGL3* involved in floral transition may correlate to delay in bolting or absence of floral organs on infected plants. Differential expression of these genes was detected only in the Arabidopsis–CLps system, and has not been reported for citrus-HLB

or psyllid yellows. Based on the phenotype and transcriptional changes, it is evident that *Arabidopsis* launches basal defense but it fails to launch adequate defense responses to CLps infection.

CONCLUSION

Overall analysis of gene expression profiles showed that *Arabidopsi*s responses were altered in ways similar to citrus infected by HLB, but it also showed some unique developmental changes to Liberibacter. Based on the transcriptional and phenotype analysis, *Arabidopsis* demonstrates a potential to contribute to our understanding Liberibacter related responses. This study offers not only a detailed insight into responses of a model plant species to the Liberibacter pathogen, but also offers a resource for future analysis of candidate genes that can aid in HLB research. Numerous mutants, and other genomic resources of *Arabidopsis* can be extremely useful to dissect and understand the mechanisms by which specific genes influence responses to Liberibacter.

Supplementary information

Table S2.1: Primer sequences for genes used in validation of microarray data by qRT-PCR.

Gene ID	Locus ID (TAIR)	Forward primer sequence	Reverse primer sequence
ATKT1	AT1G73260	CCCGAATCACAGAACCTCAA	CTTCCTCTCGTGGTCAAACTC
ATOSM34	AT4G11650	GAACGGTCAGGGATCATGTAG	GTCGGGTCATCTTGTGGATAG
ATBGLU30	AT3G60140	GTCGTCGGAGAGAGTAACAAAG	TGCCATGAAAGATGCAAAGAAA
ATCSD1	AT1G08830	CATTGGTCTCCAGGGCTAAA	GCCACACACCAGAAGATACA
ATPR3	AT3G12500	GCAAACGCTACTACGGAAGA	AAGGTCAGGGTTGTTGAGTAAG
ATDMR6	AT5G24530	GTCGAGCCTTCCTTCCTTTATC	ACCGGTGGATATCAGCTTTG
PR2	AT3G57260	CGCCCAGTCCACTGTTGATA	ACCACGATTTCCAACGATCC
PR5	AT1G75040	CCTGCAAGAGTGCCTGTGAG	GCTATAGGCGTCAGGGCAAG
ATSARP	AT4G00880	CGATCCCTTGTCACGTTGAG	CACCGGATGAGATGGTTGTG
ATAGL3	AT2G03710	CCCTCCAATTCAGGAAGCAG	AGTTGGTTGCATTTGCAGGA
ATAGL42	AT5G62165	CGAACGCTACCGCAAGTACA	GTCCCAATAGCTTCCGCTTG
AtGLUR	AT2G47880	ATCGACAACGACCCGGACTG	CTTGCCTCCGACAAAGACAGC
JAL22	AT2G39310	TGAGGGAAAGAAGATCGTAGGG	GGAACGGCGTGGTATCCAAT
UBQ10	AT4G05320	TGCGTCTTCGTGGTGGTTTC	TCAGGGATTATACAAGGCCCCA
ACT2	AT3G18780	CACATTCCAGCAGATGTGGATCTC	ACCCCAGCTTTTTAAGCCTTTG
Ef1	AT5G60390	AGGAGTTGTGTTACAAAGTTGCTGT	AACGCTAAGTATGGTTGGGTTA
UBP22	AT5G10790	GGGCTGATGTTGTTGCGGTT	ACGTCCATTTCTCCCTTCGATCT

Table S2.2: List of differentially expressed genes in Col-0 at 8 wpi in response to CLps infection.

Locus ID	Gene description	Log_2 Fold change	Adj. p-value
	Up-regulated genes in Col-0		
Response to stress / response to biotic/abiotic stimuli			
AT1G73260	Kunitz trypsin inhibitor 1	6.12	7.31E-05
AT4G11650	Osmotin 34	5.67	2.73E-05
AT3G57260	Beta-1, 3-glucanase 2 (PR2)	4.88	1.50E-04
AT1G80160	Lactoylglutathione lyase / glyoxalase I family protein	4.35	7.23E-03
AT3G04720	Pathogenesis-related 4	3.77	4.22E-02
AT2G18660	Plant natriuretic peptide A	3.75	2.72E-06
AT5G13080	WRKY DNA-binding protein 75	3.72	1.50E-04
AT4G23680	Polyketide cyclase/dehydrase and lipid transport superfamily protein	3.68	3.76E-02
AT3G18250	Putative membrane lipoprotein	3.68	3.91E-03
AT3G01970	WRKY DNA-binding protein 45	3.66	6.26E-04
AT2G14560	Protein of unknown function (DUF567)	3.62	5.91E-04
AT4G37370	Cytochrome P450, family 81, subfamily D, polypeptide 8	3.57	1.76E-02
AT1G03220; AT1G03230	[AT1G03220, Eukaryotic aspartyl protease family protein]; [AT1G03230, Eukaryotic aspartyl protease family protein]	3.57	3.92E-02
AT3G22231	Pathogen and circadian controlled 1	3.55	7.37E-03
AT5G43580	Serine protease inhibitor, potato inhibitor I-type family protein	3.36	1.23E-03
AT4G16260	Glycosyl hydrolase superfamily protein	3.13	7.35E-03
AT5G10760	Eukaryotic aspartyl protease family protein	3.12	5.38E-03
AT3G16450	Mannose-binding lectin superfamily protein	3.11	3.61E-02
AT1G03850	Glutaredoxin family protein	3.09	2.73E-05
AT2G40750	WRKY DNA-binding protein 54	3.03	2.82E-03
AT1G75040	Pathogenesis-related gene 5	3.02	2.87E-04
AT3G01420	Peroxidase superfamily protein	3.02	4.49E-02
AT2G43570	Chitinase, putative	2.96	7.08E-04
AT4G00700	C2 calcium/lipid-binding plant phosphoribosyltransferase family protein	2.95	5.98E-03
AT3G02480	Late embryogenesis abundant protein (LEA) family protein	2.92	3.23E-02
AT3G12500	Basic chitinase (PR3)	2.91	2.05E-03
AT3G26830	Cytochrome P450 superfamily protein, PHYTOALEXIN DEFICIENT 3	2.90	2.75E-05
AT1G23120	Polyketide cyclase/dehydrase and lipid transport superfamily protein	2.84	2.87E-04
AT1G55920	Serine acetyltransferase 2; 1	2.77	4.33E-02

Locus ID	Gene description	Log_2 Fold change	Adj. p-value
AT5G24530	2-oxoglutarate (2OG) and Fe (II)-dependent oxygenase superfamily protein, DMR6	2.76	6.63E-03
AT2G29350	Senescence-associated gene 13	2.73	6.39E-03
AT3G09940	Monodehydroascorbate reductase	2.55	6.99E-03
AT5G22570	WRKY DNA-binding protein 38	2.46	2.64E-03
AT2G30140	UDP-Glycosyltransferase superfamily protein	2.42	2.84E-02
AT4G3753; AT4G37520	[AT4G37530, AT4G37520 Peroxidase superfamily protein	2.40	2.24E-02
AT3G4912; AT3G49110	[AT3G49120, peroxidase CB]; [AT3G49110, peroxidase CA]	2.28	2.87E-04
AT2G14610	Pathogenesis-related gene 1	2.16	2.63E-04
AT3G47480	Calcium-binding EF-hand family protein	2.08	1.94E-02
AT5G22540	Plant protein of unknown function (DUF247)	2.05	4.00E-03
Developmental processes			
AT3G04070	NAC domain containing protein 47 (ANAC047)	4.21	3.33E-03
AT1G17020	Senescence-related gene 1	4.05	8.80E-03
AT4G01430	Nodulin MtN21 /EamA-like transporter family protein	3.25	1.22E-02
AT2G45210	SAUR-like auxin-responsive protein family	3.04	3.13E-02
AT1G56010	NAC domain containing protein 1	2.20	4.19E-03
Transcription			
AT3G01970	WRKY DNA-binding protein 45	3.66	6.26E-04
Signal Transduction			
AT1G32350	Alternative oxidase 1D	2.44	3.32E-02
Other cellular processes/metabolic processes			
AT3G60140	Glycosyl hydrolase superfamily protein		
AT5G49360	Beta-xylosidase 1	5.04	1.60E-02
AT1G15380	Lactoylglutathione lyase / glyoxalase I family protein	4.25	2.77E-02
AT1G30700	FAD-binding Berberine family protein	4.10	2.68E-03
AT4G25000	Alpha-amylase-like	3.73	5.39E-03
AT5G56870	Beta-galactosidase 4	3.46	1.32E-02
AT5G07440	Glutamate dehydrogenase 2	3.45	1.58E-03
AT4G01870	tolB protein-related	3.27	6.23E-03
AT1G67980	Caffeoyl-CoA 3-O-methyltransferase	2.99	1.99E-02
AT4G2183; AT4G21840	[AT4G21830, methionine sulfoxide reductase B7]; [AT4G21840, methionine sulfoxide reductase B8]	2.99	2.73E-04
AT4G10500	2-oxoglutarate (2OG) and Fe (II)-dependent oxygenase superfamily protein	2.93	3.16E-03
AT1G09240	Nicotianamine synthase 3	2.91	1.93E-02

Locus ID	Gene description	Log_2 Fold change	Adj. p-value
AT2G19800	Myo-inositol oxygenase 2	2.90	2.03E-02
AT1G77760	Nitrate reductase 1	2.79	4.26E-02
AT2G44790	Uclacyanin 2	2.79	2.21E-03
AT5G13330	Related to AP2 6l	2.49	5.91E-04
AT1G69930	Glutathione S-transferase TAU 11	2.35	2.01E-04
AT1G33030	O-methyltransferase family protein	2.31	3.40E-02
AT1G74590	Glutathione S-transferase TAU 10	2.21	2.10E-04
AT3G18280	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein	2.09	8.91E-03
AT2G25450	2-oxoglutarate (2OG) and Fe (II)-dependent oxygenase superfamily protein	2.09	2.95E-02
AT1G13750	Purple acid phosphatases superfamily protein	2.06	1.50E-04
AT3G26820 AT3G26840	AT3G26820, AT3G26840: Esterase/lipase/thioesterase family protein	2.02	3.89E-02
Transport			
AT4G15610	Uncharacterized protein family (UPF0497), CASP-LIKE PROTEIN 1D1	3.78	1.30E-02
AT4G35060	Heavy metal transport/detoxification superfamily protein	3.09	5.94E-03
AT5G05960	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein	2.73	3.92E-02
AT5G47560	Tonoplast dicarboxylate transporter	2.59	4.18E-02
AT4G20110	VACUOLAR SORTING RECEPTOR 7	2.46	6.33E-03
AT4G10380	NOD26-like intrinsic protein 5	2.33	8.11E-03
AT5G46050	Peptide transporter 3	2.28	5.91E-04
Unknown biological processes			
AT2G39310	Jacalin-related lectin 22	3.82	2.87E-04
AT2G23170	Auxin-responsive GH3 family protein	3.81	1.35E-02
AT3G18250	Putative membrane lipoprotein	3.68	3.91E-03
AT1G66690 AT1G66700	AT1G66690, AT1G66700: S-adenosyl-L-methionine-dependent methyltransferases superfamily protein]	3.55	6.50E-04
AT4G28040	Nodulin MtN21 /EamA-like transporter family protein	3.15	9.10E-03
AT1G19960	BEST Arabidopsis thaliana protein match is: transmembrane receptors (TAIR: AT2G32140.1).	3.15	1.75E-03
AT1G78850 AT1G78860	D-mannose binding lectin protein with Apple-like carbohydrate-binding domain	3.12	3.36E-02

Locus ID	Gene description	Log_2 Fold change	Adj. p-value
AT3G14060	Unknown protein	2.84	1.13E-02
AT2G37750	Unknown protein	2.71	2.14E-02
AT5G39670	Calcium-binding EF-hand family protein	2.49	6.33E-03
AT1G65500	Unknown protein	2.43	8.09E-03
AT1G19530	Unknown protein	2.34	6.21E-03
AT1G65510	Unknown protein	2.58	3.33E-03
AT3G22240	Unknown protein	2.28	2.02E-02
AT3G61990	S-adenosyl-L-methionine-dependent methyltransferases superfamily protein	2.06	5.38E-03
AT2G16700	Actin depolymerizing factor 5	2.01	2.20E-02
	Down-regulated genes in Col-0		
Developmental Processes			
AT5G16715	ATP binding;valine-tRNA ligases;aminoacyl-tRNA ligases;nucleotide binding;ATP binding;aminoacyl-tRNA ligases	-1.044	3.39E-02
AT5G05740	Ethylene-dependent gravitropism-deficient and yellow-green-like 2	-1.132	3.40E-02
AT4G14510	CRM family member 3B	-1.172	3.33E-02
AT2G33810	Squamosa promoter binding protein-like 3	-1.289	4.34E-02
AT4G24930	Thylakoid lumenal 17.9 kDa protein, chloroplast	-1.465	7.27E-03
AT2G03710	K-box region and MADS-box transcription factor family protein	-1.539	3.40E-03
AT5G60910	AGAMOUS-like 8	-1.897	3.15E-02
AT5G62165	AGAMOUS-like 42	-1.906	7.56E-03
AT2G30540	Thioredoxin superfamily protein	-2.378	4.57E-02
AT2G47880	Glutaredoxin family protein	-3.186	2.22E-03
Transport			
AT1G01790	K+ efflux antiporter 1	-1.406	1.54E-02
AT5G19500	Tryptophan/tyrosine permease	-1.049	2.03E-02
Other cellular processes/metabolic processes			
AT1G31800	Cytochrome P450, family 97, subfamily A, polypeptide 3	-1.080	2.03E-02
AT1G56500	haloacid dehalogenase-like hydrolase family protein	-1.107	4.25E-03
AT1G06690	NAD(P)-linked oxidoreductase superfamily protein	-1.091	6.25E-03
AT1G61580	R-protein L3 B	-1.138	4.18E-02
AT4G33500	Protein phosphatase 2C family protein	-1.145	3.35E-02
AT1G51440	Alpha/beta-Hydrolases superfamily protein	-1.166	2.68E-02
AT5G62840	Phosphoglycerate mutase family protein	-1.216	3.23E-02
AT1G29720	Leucine-rich repeat transmembrane protein kinase	-1.248	1.95E-02

Locus ID	Gene description	Log_2 Fold change	Adj. p-value
AT5G22620	phosphoglycerate/bisphosphoglycerate mutase family protein	-1.440	8.11E-03
AT5G09300	Thiamin diphosphate-binding fold (THDP-binding) superfamily protein	-1.445	1.78E-02
AT1G15410	Aspartate-glutamate racemase family	-1.513	4.13E-02
AT2G29450	Glutathione S-transferase tau 5	-1.594	4.39E-02
AT3G28500	60S acidic ribosomal protein family	-1.813	2.23E-03
Response to stress			
AT1G44970	Peroxidase superfamily protein	-1.588	7.43E-03
Transcription			
AT1G72050	Transcription factor IIIA	-1.46	1.00E-02
Other biological processes			
AT5G62000	Auxin response factor 2	-1.113	3.26E-02
AT4G00880	SAUR-like auxin-responsive protein family	-1.444	1.99E-02
Unknown Biological processes			
AT5G52070	Agenet domain-containing protein	-1.001	1.40E-02
AT1G79510	Uncharacterized conserved protein (DUF2358)	-1.013	1.70E-02
AT4G00780	TRAF-like family protein	-1.169	1.90E-02
AT3G57630	exostosin family protein	-1.255	8.80E-03
AT4G28740	Unknown protein	-1.298	6.23E-03
AT1G67865	Unknown protein	-1.758	8.80E-03
AT3G62820	Plant invertase/pectin methylesterase inhibitor superfamily protein	-1.671	3.92E-02
AT1G64360	Unknown protein	-1.914	8.11E-03
AT5G58310	Methyl esterase 18	-2.359	2.68E-02

Table S2.3: List of differentially expressed genes in *NahG* at 8 wpi in response to CLps infection.

Locus ID	Gene description	Log_2 Fold change	Adj. p-value
	Up-regulated genes in NahG		
Response to stress / response to biotic/abiotic stimuli			
AT3G57260	Beta-1, 3-glucanase 2	5.557	2.40E-05
AT2G18660	Plant natriuretic peptide A	4.640	7.80E-08
AT3G26830	Cytochrome P450 superfamily protein, Phytoalexin deficient 3	4.209	1.28E-07
AT5G10760	Eukaryotic aspartyl protease family protein	4.017	8.51E-04
AT3G16450	Mannose-binding lectin superfamily protein	3.938	1.46E-02
AT1G75040	Pathogenesis-related gene 5	3.791	2.40E-05
AT2G44790	Uclacyanin 2	3.577	2.23E-04
AT1G73260	Kunitz trypsin inhibitor 1	3.536	1.25E-02
AT3G12500	Basic chitinase (PR3)	3.311	8.51E-04
AT4G16260	Glycosyl hydrolase superfamily protein	3.119	1.39E-02
AT5G39670	Calcium-binding EF-hand family protein	3.042	2.07E-03
AT1G19250	Flavin-dependent monooxygenase 1	2.81	8.59E-07
AT4G11650	Osmotin 34	2.685	2.47E-02
AT2G43570	Chitinase, putative	2.451	5.89E-03
AT1G70850	MLP-like protein 34	2.426	6.47E-03
AT3G47480	Calcium-binding EF-hand family protein	2.407	1.35E-02
AT4G36430	Peroxidase superfamily protein	2.400	2.07E-03
AT5G59720	Heat shock protein 18.2	2.374	2.04E-02
AT2G30750	Cytochrome P450, family 71, subfamily A, polypeptide12	2.371	2.07E-03
AT1G76930	Extensin 4	2.334	1.62E-02
AT1G35230	Arabinogalactan protein 5	2.292	1.46E-02
AT5G13320	Auxin-responsive GH3 family protein	2.116	4.77E-03
AT1G35260	MLP-like protein 165	2.027	1.46E-02
Other cellular processes/metabolic processes			
AT4G21830	Methionine sulfoxide reductase	3.586	2.40E-05
AT5G17330	Glutamate decarboxylase	2.89	2.88E-06
AT3G54260	TRICHOME BIREFRINGENCE-LIKE 36	2.762	3.20E-02
AT2G22170	Lipase/lipooxygenase, PLAT/LH2 family protein	2.642	4.05E-02
AT1G67030	Zinc finger protein 6	2.637	5.23E-04
AT5G07440	Glutamate dehydrogenase 2	2.421	3.35E-02
AT5G04950	Nicotianamine synthase 1	2.289	2.23E-04
AT5G44400	FAD-binding Berberine family protein	2.103	2.03E-02
Transport			
AT5G05960	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein	2.73	3.92E-02

Locus ID	Gene description	Log_2 Fold change	Adj. p-value
Unknown biological processes			
AT1G66690; AT1G66700	S-adenosyl-L-methionine-dependent methyltransferases superfamily protein	3.54	6.50E-04
AT2G39310	jacalin-related lectin 22	4.72	2.40E-05
AT1G65500	Unknown protein).	4.12	6.86E-05
AT5G39110; AT5G39150; AT5G39120; AT5G39180	RmlC-like cupins superfamily protein	2.41	1.17E-03
Down-regulated genes in NahG			
Developmental processes			
AT2G03710	K-box region and MADS-box transcription factor family protein	-1.420	9.32E-03
AT5G60910	AGAMOUS-like 8	-1.933	4.78E-02
AT5G62165	AGAMOUS-like 42	-2.170	4.77E-03
AT2G47880	Glutaredoxin family protein	-2.388	2.86E-02
Transport			
AT3G53720	Cation/H+ exchanger 20	-1.361	2.68E-02
AT2G36590	Proline transporter 3	-1.632	5.89E-03
Other cellular processes/metabolic processes			
AT2G23390	Unknown protein	-1.070	3.20E-02
AT1G19630	Cytochrome P450, family 722, subfamily A, polypeptide 1	-1.136	3.28E-02
AT5G09300	Thiamin diphosphate-binding fold (THDP-binding) superfamily protein	-1.567	1.88E-02
AT4G10120	Sucrose-phosphate synthase family protein	-2.000	2.38E-02
Response to stress			
AT1G64360	Unknown protein	-1.988	1.13E-02
Protein Metabolism			
AT3G27110	Peptidase family M48 family protein	-1.398	7.16E-03
Other biological processes			
AT4G00880	SAUR-like auxin-responsive protein family	-1.446	4.00E-02
Unknown Biological processes			
AT4G00780	TRAF-like family protein	-1.414	8.51E-03
AT1G67865	Unknown protein (source: NCBI BLink).	-2.191	2.64E-03
AT1G34040	Pyridoxal phosphate (PLP)-dependent transferases superfamily protein	-3.352	3.28E-02

CHAPTER 3: Investigation of foliar spray technique and detached leaf assay for screening and identification of defense inducing compounds in tomato, *Arabidopsis* and citrus.

SUMMARY:

Despite the extensive research efforts on Huanglongbing disease, inherent challenges associated with citrus species such as longer generation time, incompatibility issues and lack of efficient genomic resources delay the development of control strategies. In this study, an approach of testing small molecular weight chemical compounds with a model plant-vector-pathogen system was examined to determine its potential in rapid development of a control strategy for HLB disease. This strategy is based on a chemical genomics approach in which high throughput chemical screening is conducted to identify chemicals inducing a desired biological effect. Elucidating the mechanism of such defense inducing chemicals and their respective interacting proteins can enable researchers to design strategies wherein protein functions can be modulated by transgenic or other methods to produce desired defense effects. Many such defense-inducing compounds are non-biocidal, thereby making them great alternatives for routinely used pesticides. In this study, I investigated two chemical treatment systems (detached leaf and foliar spray) for their ease and consistency in producing CLps positive leaves or plants, the feasibility of chemical application and validation of chemical uptake. The reproducibility of both systems was crucial in the development of a

chemical screening assay with a model plant-vector-pathogen system. In detached leaf assay, a single detached tomato leaf petiole was incubated in a solution and the leaflets were exposed to *Candidatus* Liberibacter psyllaurous (CLps) positive adult psyllids. This assay generated a consistent and high percentages of CLps infected tissues in repeat experiments. In the foliar spray assay, chemicals were sprayed on soil grown *Arabidopsis* plants. Candidate chemical compounds (DCA, CMP442, CMP 199, CMP 508, CMP 974 and CMP 815) found to be effective in triggering plant defenses in other pathosystems were tested with the *Arabidopsis*-CLps system. In the preliminary screen, CMP442 and DCA treatments had either low CLps genome numbers or absence of infected plants, and therefore these compounds were retested with more replicates for validation of effects. In the validation experiment, 25 µM CMP442 and 25 µM DCA treated plants showed less bacterial growth (statistically not significant) in comparison to mock DMSO treatment, but no difference in symptoms was observed between DMSO and chemically treated CLps positive plants. In a different study, no effect was observed on CLps transmission and related phenotype when plants were treated with CMP442 before infestation. In all experiments, chemical uptake by plants was confirmed by induction of a reporter gene, *CaBP22*. In addition to *Arabidopsis*, chemicals DCA, CMP442 were sprayed on citrus to determine if similar changes are induced. Expression of defense pathway genes such as *PR1, LURP1* and *WRKY70* was analyzed 18 hrs post treatment and no gene showed significant differential expression in response to chemical treatment.

ABBREVIATIONS:

CLps – *Candidatus* Liberibacter psyllaurous, SA- Salicylic acid, JA-Jasmonic acid, BC – Bactericera cockerelli, wpi- weeks post infestation, *ein2-5*- ethylene insensitive 2-5.

INTRODUCTION

Control measures for vector-transmitted plant diseases are usually dependent on eradication of insect vectors by pesticides or other chemical treatments. Pesticides can be efficient in controlling insect population but these measures are not always effective in controlling spread of the disease-causing pathogen. Even with wide use of pesticides, Huanglongbing (HLB), a vector-transmitted bacterial disease has spread in to almost all citrus growing counties of Florida (Brlansky et al., 2014b). HLB is associated with three phloem-inhabiting strains of bacteria known *Candidatus Liberibacter* asiaticus (CLas) (Jagoueix et al., 1994), *Candidatus* Liberibacter americanus (CLam) (Texeira et al., 2005) and *Candidatus* Liberibacter africanus (CLaf) (Jagoueix et al., 1994). CLas and CLam are transmitted into citrus trees by the hemipteren psyllid, *Diaphorina citri* (Kuwayama) (Capoor et al., 1967; Texeira et al., 2005) also commonly known as Asian citrus psyllid and CLaf is transmitted by *Trioza erytreae* Del Guercio (McClean and Oberholzer 1965). At an early stage of infection, HLB infected trees show symptoms including leaf chlorosis or asymmetric discoloration on leaves (McClean and Schwarz, 1970), however as infection spreads, trees drop leaves, twigs or shoots tend to die back, and fruits remain green and are smaller in comparison to fruits from healthy trees (Da Graca, 1991). In severely infected trees, young fruits fail to mature, tend to drop early and fruit juices are bitter in taste (Baldwin et al., 2010). In the absence of any treatment or control

measure, HLB infected citrus groves can become completely unproductive. Studies demonstrate the potential of antibiotic applications, chemical treatments and thermotherapy in eliminating HLB pathogen (Hoffman et al., 2013; Puttamuk et al., 2014; Zhang et al., 2011, 2014). Hoffman's study (Hoffman et al. 2013) shows that CLas-infected citrus seedlings grown in controlled growth chambers and exposed to a temperature of 42^{O}C over variable periods of 2-10 days can significantly reduce bacterial titer or can eliminate it in aboveground parts of the tree. A separate study (Duan and Doud, 2014) was conducted wherein the heat treatment was applied to trees in fields. In this study, the treatment was not completely effective in eliminating CLas from all trees in fields as it was shown in controlled environment conditions. In some cases bacteria returned after 2 years of treatment. The factors that reduce the effectiveness of thermal therapy are variability in environmental conditions in fields, severity of pathogen infection in trees (heat treatment was unable to eliminate CLas from severely infected trees) and the possibility that the bacteria residing roots can re-infect the scion. Also, these field trials were unable confirm a consistent positive effect of continuous heat application on plant growth, fruit production and overall tree health in all *Citrus* varieties (Duan and Doud, 2014). The cost-effectiveness of building tents and variable responses of citrus varieties can also affect the potential use of thermotherapy for controlling HLB disease in fields. Disease management by application of antibiotics such as penicillin, streptomycin, tetracycline, was shown to suppress HLB symptoms and reduce CLas titer for few months (Puttamuk et al. 2014). Small and long-term side

effects of residual concentrations of antibiotic in fruits, regulatory approval and consumer acceptance of fruit from such antibiotic-treated trees needs to be determined before a wide spread application of such technique. So far, thermotherapy and chemical treatment are effective on HLB disease to certain extent, but they also pose significant disadvantages, among which are the relatively short duration of reduced pathogen level, dependency on consistent and continuous application of treatments to reduce bacterial titer and unknown long term effects of antibiotics or other chemicals used in eliminating pathogen. Commonly used strategies to control spread of HLB disease in Florida and California are the use of insecticides to control spread of psyllids and applying quarantine regulations to avoid movement of HLB infected trees into new zones. Studies on application of the insecticide Imidacloprid at sub-lethal concentration (0.1 µg/ml) show that Imidacloprid can not only affect citrus psyllid survival, development and reproduction but also impacts psyllid feeding on citrus trees (Boina et al., 2009). Although applying pesticides is a preferred approach by farmers to protect food crops from diseases, there are several risks associated with long-term application. The mechanism by which this approach typically functions to eliminate pests is by interacting with nerve targets of pests such as acetyl cholinesterase, the voltage-gated chloride channel, the acetylcholine receptor, and the γ -aminobutyric acid receptor (Casida, 2009) and can affect other organisms. Imidacloprid has been indicated as highly toxic to pollinators like bees when applied during bloom time. Along with these off target effects, concerns of consumers over lingering pesticide residues (Damalas and

Eleftherohorinos, 2011), provide evidence that use of pesticides is not very desirable. Eliciting innate defenses of plants to control disease would be preferred over applying toxic chemicals. Chemical genetics or genomics approach has been demonstrated to be useful in study of plant-pathosystem and eliciting defense in plants (Knoth et al., 2009; Schreiber et al., 2008).

Chemical Genomics

Chemical genomics uses small molecules to probe desired biological processes with genomic-scaled data such as protein or gene sequences, structure and functions. This approach is based on the principle that small molecules interact with proteins (or other ligands) and modulate their activity in a reversible or tunable manner (Zheng et al., 2004). Bioactive small molecules can probe redundant gene functions by modulating activity of related gene products simultaneously. Application of small molecules in varying concentrations can be useful in modulating gene products to a desired concentration providing an advantage by overcoming the lethal phenotype resulting from some homozygous knockout mutations (Raikhel and Pirrung 2005). Genetic mutation is permanent and often reduces our ability to understand genes expressed at specific stages, but small molecules can be applied transiently to study role of such genes (Raikhel and Pirrung 2005). These small molecules are considered as “druglike” when they meet requirements such as low molecular weight, have an ability to cross biological membranes and remain (biologically) active for an adequate period (Hopkins

and Groom, 2002). Lipinski “rule of five” is used to determine if a compound is likely to be “drug like”. The rule of five states that a small molecule may have poor absorption or permeation if its molecular weight is more than 500, lipophilicity (CLogP) is greater than 5, and when there are more than 5 Hydrogen bond donors and 10 H bond acceptors (Lipinski et al., 1997).

Application of Chemical Genomics in Plant Research

There are usually three stages in a chemical genomics approach for studying any biological process: 1) development of a high-throughput chemical screening assay, 2) selection, screening of chemical compounds and validation of compounds inducing desired effects by performing repeat screening, 3) investigation of target protein and small molecule interactions (Robert et al., 2009). The structural and chemical diversity of target proteins for binding chemicals has influenced combinatorial chemistry based synthesis of chemical libraries useful for probing protein functions. These synthesized chemical libraries are classified into two types, diversity-oriented and focused types (Young and Ge, 2004). Focused type libraries have compounds that bind to a defined class of proteins, whereas the diversity-oriented libraries contain compounds with diverse chemical structures that can target different protein classes (Stockwell, 2004). Albeit the use of chemical genomics in plant research is recent, several studies show success in identification of small molecules interacting with different target proteins in various biological processes (Armstrong et al., 2004; Park et al., 2009; De Rybel et al.,

2009b; Savaldi-Goldstein et al., 2008; Surpin et al., 2005). Along with the biochemical characterization of desired interacting proteins, small molecules can also be useful to boost plant immunity. Sticher's review (Sticher et al., 1997) on systemic acquired resistance (SAR) discusses several studies that demonstrate that exogenous application of hormones or related compounds can enhance plant defense responses. Pathogenesis related proteins were induced by exogenous application of salicylic acid (SA) as well as methyl-2,6-dichloroisonicotinicacid (INA) (Ward et al., 1991) triggering resistance to bacterial and fungal pathogens in *Arabidopsis* (Uknes et al., 1992). Benzo(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH) activates SAR in tobacco without accumulation of SA (Friedrich et al., 1996) and induces resistance to powdery mildew in wheat (Görlach et al., 1996). Jasmonic acid and it derivatives are also known to induce resistance pathways in many plant species. Treatment of first leaves of barley with methyl jasmonate activated systemic defense to powdery mildew (Walters et al., 2002). Brassinosteriod signaling pathways are involved in various processes such as plant development and stress related responses. Use of 24-epibrassinolide (Brassinosteriod) induced tolerance to abiotic stress in *Arabidopsis* seedlings (Divi et al., 2010). Synthetic defense inducers such Tidanil, and Probenazole are known to induce Pathogenesis related proteins (PR) genes and trigger SAR (Nakashita et al., 2002; Watanabe, 1977; Yasuda et al., 2004). To identify candidate chemical compounds that induce defense responses, usually high throughput screens with thousands of chemical compounds are conducted. From 3000-member LATCA library, a chemical compound Sulfamethoxazole

(SMEX) was identified that reduced *Pseudomonas syringae* growth in *Arabidopsis* seedlings (Schreiber et al., 2008). Successful application of high throughput chemical screening to identify defense inducers was presented in Knoth's study (Knoth et al., 2009). In this study (Knoth et al., 2009), 114 synthetic defense elicitors were identified in the *Arabidopsis* and *Hyalopernospora parasitica* pathosystem. Among these several compounds, 3,5-Dichloroanthranilic acid (DCA) (Knoth et al., 2009), CMP442 and CMP 199 (Rodriguez-Salus, 2012) are suggested to be potent defense inducers and induce defenses via the SA pathway. We tested the effect some of these chemical compounds with the *Arabidopsis thaliana* –psyllid yellows plant pathosystem.

Arabidopsis **and Psyllid Yellows**

"Psyllid yellows" is a yellowing condition associated with phloem-limited bacteria known as *Candidatus* Liberibacter psyllaurous (Hansen et al., 2008). This bacterium can infect solanaceous plants such as *Solanum lycopersicon*, *Solanum tuberosum*, and *Capsicum annum. Candidatus* Liberibacter psyllaurous is also known as *Candidatus* Liberibacter solanacearum (Liefting et al., 2009), is transmitted by a hemipteren psyllid, *Bactericera cockerelli* (Sulc) (BC). Infected plants develop symptoms such as leaf chlorosis, purpling along midveins, shorter internodes, leaf cupping, lower fruit production or small size fruits and overall stunted growth of plants (Cranshaw, 1994; Liefting et al., 2009; Pletsch, 1947). There are several similarities in the mode of transmission, *16S rDNA* sequences, and disease symptomology of the pathogens associated with HLB and psyllid

yellows. In preliminary studies, we found that *Bactericera cockerelli* (Sulc) transmits CLps into *Arabidopsis thaliana* plants, however the psyllid was unable to complete its lifecycle on *Arabidopsis*. Analysis of responses of genetically diverse ecotypes and defense mutants of *Arabidopsis thaliana* to CLps infection indicate that bacterial titer increases over time and plants eventually develop symptoms in response to infection (Chapter 1). All lines screened for CLps resistance had some plants detected positive for CLps infection and at 5-6 wpi under short day conditions, and infected plants displayed distinct symptoms such as distinct leaf discoloration, leaf curling, excessive small size axillary leaves, and delayed stem development.

The objective of this study was to explore different assays that could be used to conduct chemical screening for defense-inducing chemicals effective against bacteria Liberibacter species. A tomato detached leaf assay based on a citrus leaf assay used by Ammar et.al (Ammar et al., 2013) was investigated for screening of chemical compounds with tomato. Application of chemicals to soil-grown *Arabidopsis* seedlings using a foliar spray technique was also tested. Candidate chemical compounds DCA (3,5-Dichloroanthranilic acid) and CMP442 (2-(5-bromo-2- hydroxyphenyl)-Thiazolidine-4-carboxylic acid) were found to be effective in other plant defense studies (Knoth et al. 2009; Rodriguez-Salus 2012). In this study, these compounds along with other defense elicitors were tested with soil grown *Arabidopsis thaliana* infected with *Candidatus* Liberibacter psyllaurous. Instead of the *NahG* and Col-0 lines that were previously studied for CLps infection related phenotype and genetic responses (Chapter 2), we

have used the *ein2-5* line to conduct chemical screening. Our experimental results indicate that Col-0 line shows considerable variability in both percentage of CLps positive plants and bacterial titer. Based on previous experimental observations with *ein2-5* line infected plants, there was consistency in the percentage of CLps positive plants detected by leaf tissue testing and plants had high bacterial titer in leaf tissues unlike Col-0. The *ein2-5* line had higher percentage of CLps positive plants at 3 wpi compared to wild type Col-0 line. The frequency analysis of symptoms in all lines (Chapter 1) showed that CLps-infected plants of *ein2-5* line had strong association with symptoms such as discoloration, leaf curling and axillary/cauline leaf growth in comparison to other lines at 6 wpi. Our key objective in chemical screening was to investigate if any chemical shows potential in eliciting plant defense in response to CLps infection. The selection of *ein2-5* line over *NahG* line was based on two criteria 1) the potential mode of action of selected candidate compounds and 2) screening an adequate number of compounds to demonstrate the potential of *Arabidopsis* in conducting chemical screening with psyllid yellows. The *ein2-5* mutation disrupts the ethylene pathway and *NahG* transgene affects the SA pathway. Knoth's study showed that the mode of action of chemical compound DCA is downstream of the SA pathway and is partially dependent on the genes *NPR1* and *WRKY70* (Knoth et al. 2009). In the *NahG* line, SA is converted to catechol and as a result it fails to activate SA related responses. The chemical compound DCA was able to trigger full resistance via SA pathway in *NahG* plants to *Hyalopernospora parasitica* infection (Knoth et al. 2009)

whereas CMP442 was not able to induce any resistance in *NahG* against *Hyalopernospora parasitica* (Rodriguez-Salus 2012) indicating that the mode of action of CMP442 is different from that of DCA and upstream of the SA pathway. In this scenario, to include more candidate chemicals, the *ein2-5* line (which has a functional SA pathway) was considered more suitable than the *NahG* line. Previous comparison of responses to CLps infection showed no significant difference in the percentage of CLps infected plants (60 and 53.33), bacterial genomes / plant cell (48 and 43) and symptoms between *NahG* and *ein2-5* lines respectively (Chapter 1). Along with investigating defense responses to CLps, effectiveness of the foliar spray technique was confirmed by validating chemical uptake in plants. Chemical uptake by *Arabidopsis* plants was confirmed by expression analysis of *CaBP22* gene, a calmodulin-like calcium binding protein (McCormack et al., 2005). *CaBP22* is one of the genes belonging to the cluster of Late Up-regulated in Response to *Hyaloperonospora parasitica* (*LURP*) genes. Microarray analysis showed that *CaBP22* was up regulated at 12hr-48hr post *Hyalopernospora parasitica* infection (Eulgem et al., 2004) as well as in response to DCA and CMP442 chemical treatments (Knoth et al. 2009; Rodriguez-Salus 2012). In addition to testing the potential of *Arabidopsis*, a small-scale chemical screening experiment was conducted with *Citrus sinensis* seedlings to determine the potential of foliar spray technique in applying small molecular weight chemical compounds to citrus. DCA and CMP442 were tested to determine if they induce the same defense genes in both *Arabidopsis thaliana* and *Citrus*.

MATERIALS AND METHODS

Plant growth conditions and psyllid infestation

Seeds of *Arabidopsis thaliana* mutant line *ein2-5* provided by Dr. Thomas Eulgem (University of California, Riverside), were sowed in pesticide/fungicide free soil and subjected to cold stratification for 72-hours for uniform germination. All plants were grown under short day conditions (8hrs day at 23°C /16hrs night at 22°C) in an environmental growth chamber. For both the preliminary and the validation screens, at 4-weeks stage, plants were divided into two groups. In one group (post-infestation chemical treatment), one plant per pot was infested with 10 nymphs (3rd-5th instar stage) obtained from CLps-infected tomato plants and at 4 weeks post infestation (wpi); plants were treated with chemicals or control sprays. In the other group (pre-infestation chemical treatment), multiple plants per pot were treated with chemicals after which all but one plant were removed for testing chemical uptake at 48 hours post treatment and one plant per pot was infested with 10 nymphs (3rd-5th instar stage). All infested and non-infested plants were covered with organza bags and then placed in separate bugdorms (BioQuip products, 1462W). In the citrus experiment, 3 months old *Citrus sinensis* (L.) Osbeck (Argentina sweet orange) seedlings were grown in greenhouse conditions in a chemical free environment for 3 months from Dec-March at temperature 20°C -25°C before foliar application of DCA, CMP442 and BTH.

Tomato detached leaf assay (TDL):

Tomato plants (*Solanum lycoperiscon* var. Moneymaker) were grown at 21-26 °C and 40-60% relative humidity in an environmental growth chamber. Leaves of similar growth stage were detached from plants at the petiole and infested with 10-15 adult *Bactericera cockerelli* psyllids. Two similar but slightly different setups were used for TDL assays. In one set up used (Fig 3.1A-B), a single leaf with a minimum 3 leaflets was placed in a 2ml microfuge tube containing water, which was then placed in 50ml tube covered with mesh cap. The microfuge tube contained a plastic straw for addition of water/chemicals. In the second design, a single leaf was placed in a vial filled with water and an attached tube. 3 vials were placed in one magenta box and each magenta box was then covered with mesh bag (Fig 3.1C). The attached tube was used for adding water and chemicals to the vials without opening the mesh bags, thus avoiding psyllid escape. Each leaf was thoroughly cleaned prior to sampling of tissues for CLps detection at 14dpi (days post infestation). Two trials were conducted using each set up (Fig 3.1A-B) with different number of replicates. Trials 1 and 2 included 6 infested detached leaves and 20 infested detached leaves respectively.

DNA isolation

The tomato plant tissue samples were transferred into 2 ml tubes containing 2.5 mm zirconium beads (Research Products International Corp.) with 400 µl of extraction buffer (200 mM Tris, (pH 7.5) 250 mM NaCl, 25 mM EDTA, 0.5% SDS). All tissue samples were homogenized in MP Biomedicals FastPrep®-24 and 400 µl of chloroform: Isoamyl alcohol (24:1) was added to separate the aqueous phase. With *Arabidopsis*, all tissue samples were transferred into 2ml tubes containing 2.5mm zirconium beads (Research Products International Corp.) with700 µl of extraction buffer (100mM Tris, (pH 7.5) 500 mM NaCl, 50mM EDTA, 2.5% SDS). All samples were homogenized for 60 seconds in a MP Biomedicals FastPrep®-24 at a setting of speed 6.5m/s and time 60 seconds. After centrifugation, isopropanol (and 0.2M potassium acetate only with *Arabidopsis* tissues) was added to the supernatant for DNA precipitation. All samples were incubated overnight at -20°C and the precipitated DNA pellets were washed with 70% ethanol. Purified DNA was dissolved in 100µl of sterile water and stored at 4°C.

Real time quantitative PCR for *Candidatus* Liberibacter psyllaurous detection:

All DNA samples extracted from tissues of infested and non-infested plants were tested for *Candidatus* Liberibacter psyllaurous infection by real time quantitative PCR using a TaqMan assay. In the TaqMan assay, all multiplex reactions were set up in 96 well plates (USA Scientific) with each reaction mixture (25 µl) containing 1X reaction buffer (10mM Tris-HCl, 50mM KCl, 1.5mM $MgCl_2$, pH 8.3, New England Biolabs), 250 µM dNTPs (Promega), 2.5 units of *Taq* DNA polymerase (New England Biolabs), 0.25 µM

each of primers Les 33 and Les 34 (CLps-pathogen), Les 37 and Les 38 (for *Arabidopsis*), and forward and reverse primers for *COX* gene (for Tomato), 0.15uM each of Liberibacter *16S rDNA* probe, *Arabidopsis ACT2* probe or Tomato *COX* gene probe (Table 3.1), 2 µl of DNA template and water to bring the volume to 25 µl. All reactions were performed in a Bio-Rad iQ5 thermal cycler with cycling conditions 95.0 °C for 10 min, followed by 40 cycles of 95.0 °C for 30 seconds and 58.0 °C for 55 seconds. For both assays, each plate contained 2 to 4 non-template controls (NTC), and *Candidatus* Liberibacter psyllaurous-positive standard controls. All NTC controls were randomly placed to detect potential cross contamination among reactions. The range of efficiency for all qPCRs was between 89%-100%. A Ct value of 33 was used as the threshold to call plants infected by CLps. The number of CLps genomes and plant cells were determined using standard curves generated for CLps *16S rDNA* (EU812559) and *Arabidopsis ACT2* gene. The standard curve for *16S* rDNA was generated with Ct values of 10-fold serial dilutions of plasmid DNA containing *16S* rDNA and the corresponding log of copy numbers of target DNA. Standard curve of *Arabidopsis ACT2* gene was generated using Ct values of 10 fold serial dilutions of a plant sample (# GT711) with DNA concentration (330ng/µl) and the corresponding log of copy numbers of target DNA. Copy number calculations for both genes are described in supplementary information of Chapter 1 (TableS1.2-Table S1.4).

Figure 3.1A-C: Tomato detached leaf assay experimental set up. **A-B)** Detached tomato leaf placed in microfuge tube and 50ml tube with a straw to add water **C)** Detached tomato leaf in vials attached with a tube placed in magenta box.

Chemical treatments

Chemicals DCA, CMP442, CMP199, CMP974, CMP508, and CMP815 were tested individually and in combinations (CMP199 + CMP442 + DCA, CMP442 + DCA + CMP974, CMP442 + DCA + CMP815). Stock solutions of chemicals (Table 3.2) were prepared in 100% DMSO and diluted with sterile water to 25 µM (or 8.3 µM for compounds in combination) concentration. 1000 µl of diluted solutions were sprayed on soil grown *Arabidopsis ein2-5* mutant plants at indicated weeks by Preval sprayers (www.prevalspraygun.com). The final concentration of DMSO in each treatment and control was 0.05%. For citrus, the chemicals were applied at 3 different concentrations of 5mM, 3mM and 1mM along with 0.01% surfactant (Widespread Max). Two leaves of

each plant were sprayed with a total of 1000 µl of chemical solution. Mock treatment was 0.5% DMSO with surfactant 0.01%.

RNA isolation and reverse transcription-PCR (RT-PCR)

Leaf tissues were harvested from plants sprayed with chemicals for confirming chemical uptake by examining gene expression of *CaBP22* gene for *Arabidopsis* and *LURP1*, *WRKY70*, and *PR1* for citrus. Total RNA was isolated from leaves using TRIzol® (Life Technologies) following the manufacturer's instructions. Total RNA concentration and quality were assessed from the ratio of absorbance readings at 260 and 280 nm using a Nanodrop ND2000 spectrophotometer (Thermo Scientific). RNA samples with concentration more than 100ng/µl and A260/A280 of 1.8-2.3 were selected for RT-PCR. For both *Citrus* and *Arabidopsis* RNA samples, the reaction mixture was prepared with 400ng of DNAse treated total RNA, 2µl of 50µM Oligo$(dT)_{20}$ (Invitrogen), 1µl of 10mM dNTP (Promega), 1µl of M-MuLV Reverse Transcriptase (New England BioLabs® Inc), 1µl of Murine RNAse inhibitor (40U/µl) (New England BioLabs® Inc), 2µl of 10X RT buffer (New England Biolabs) and final volume was made up to 20µl using nuclease free water. This reaction mixture was used for reverse transcription of RNA following manufacturer's instructions (New England BioLabs® Inc). Synthesized cDNA of *Arabidopsis* was used for amplification of loading control *ACTIN8* using reaction conditions of 94°C for 5 minutes, and 21 cycles of 95°C for 30 seconds, 60°C for 60 seconds and 72°C for 40 seconds and *CaBP22* using reaction conditions 94°C for 5

minutes, and 24 cycles of 95°C for 30 seconds, 63°C for 60 seconds and 72°C for 40 seconds. Synthesized cDNA of citrus samples was used for amplification of *GADPH*, *PR1* and *LURP1* fragments using the following reaction conditions: 95°C for 10min, 20-35 cycles of 95°C for 45 seconds, 60°C for 60 seconds, 72°C for 40 seconds and *WRKY70* using following reaction conditions 95°C for 10min, 20-35 cycles of 95°C for 0.45 minute, 62°C for 60 seconds and 72°C for 40 seconds. Aliquots of amplified products were collected at 20cycle, 25cycle, 30cycle and 35cycle time points to determine change in transcript levels for citrus. These PCR products were electrophoresed on 2% agarose gels containing 0.5-µg/ml ethidium bromide and visualized on a Bio-Rad Gel-Doc XR system.

Table 3.1 List of primers and probes used in this study.

Accession ID*	Target genes	Target Organism	Primer ID*	Sequences 5'->3'	Reference
EU812559	*16S rDNA*	CLps	Les33	TCGAGCGCTTATTTTTAATAGG	(Hansen et al., 2008)
L22532	*16S rDNA*	CLas	Les34	TGCGTTATCCCGTAGAAAAAGGTAG	(Li et al., 2006)
821411	*ACTIN2*	*Arabidopsis thaliana*	Les37	GTTGGGATGAACCAGAAGGA	This study
821411	*ACTIN2*	*Arabidopsis thaliana*	Les 38	GGCCTTTGGGTTAAGAGGAG	This study
L22532	*16S rDNA*	CLas/CLam/ CLps/CLaf	Les39	FAM-AGACGGGTGAGTA ACGCG- BHQ1	(Li et al., 2006)
821411	*ACTIN2*	*Arabidopsis thaliana*	Les41 ACT2 probe	Cy5-TTGCTCCTGAAGAG CACCCTGTTCTT-BHQ3	This study
CX297817	Cytochrome Oxidase (*COX*)	Tomato	Forward primer	GTATGCCACGTCGCATTCCAGA	(Li et al., 2006)
CX297817	Cytochrome Oxidase (*COX*)	Tomato	Reverse primer	GCCAAAACTGCTAAGGGCATTC	(Li et al., 2006)
CX297817	Cytochrome Oxidase (*COX*)	Tomato	COX probe	CY3-ATCCAGAT GCTTA CG CTGG-BHQ2	(Li et al., 2006)
NM_129674	*CaBP22*	*Arabidopsis thaliana*	Forward primer	CGGAACCATCAATTTCACTGAGT	(Knoth et al., 2009)
NM_129674	*CaBP22*	*Arabidopsis thaliana*	Reverse primer	CAAAGTGCCACCAGTTGTGTCAT	Knoth et al. 2009)
NM_103814	*ACTIN8*	*Arabidopsis thaliana*	Forward primer	ATGAAGATTAAGGTCGTGGCAC	(Knoth and Eulgem, 2008)
NM_103814	*ACTIN8*	*Arabidopsis thaliana*	Reverse primer	GTTTTTATCCGAGTTTGAAGAGGC	(Knoth and Eulgem, 2008)
XM_006486087	*WRKY70*	*Citrus sinensis*	Forward primer	TCAGCAGCAGCAGGCGAATTCTT	(Mafra et al., 2013)
XM_006486087	*WRKY70*	*Citrus sinensis*	Reverse primer	GTCCTTCGCCGCCGGTCTCT	(Mafra et al., 2013)
XM_006486759	*PR1*	*Citrus sinensis*	Forward primer	AAATGTGGGTGAATGAGAAAGC	(Mafra et al., 2013)
XM_006486759	*PR1*	*Citrus sinensis*	Reverse primer	ATTATTGTTGCACGTCACCTTG	(Mafra et al., 2013)
XM_006481079.1	*LURP1*	*Citrus sinensis*	Les54	CCTCAGTATTGCTGCCCTTAC	This study
XM_006481079.1	*LURP1*	*Citrus sinensis*	Les55	TGCTCTCTCCTCTGAACACT	This study
XM_006476919	*GADPH*	*Citrus sinensis*	Forward primer	GGAAGGTCAAGATCGGAATCAA	(Fan et al., 2011)
XM_006476919	*GADPH*	*Citrus sinensis*	Reverse primer	CGTCCCTCTGCAAGATGACTCT	(Fan et al., 2011)

*GenBank or NCBI reference ID

RESULTS

80% to 85% of detached tomato leaves were detected positive for *Candidatus* Liberibacter psyllaurous by petiole segment testing.

Like HLB, "Psyllid Yellows" is a pathosystem that involves three biological components (host plant, insect vector and pathogen) and behavior of each component affects the efficiency of studying this system by any assay. In-vitro culture methods such as the multi-well plate liquid/solid MS culture assay (routinely used in high throughput chemical screening studies) and hydroponic cultures were tested to determine the efficacy of these assays with psyllid yellows. These preliminary tests indicated that these assays do not yield a high number of CLps positive plants (10-20%) and had inconsistent numbers in replicate experiments. In chemical genomics based studies, the first stage is development of an efficient and highly repeatable assay with desired organisms. However, in our study, inability to produce a high proportion of CLps positive plants hindered the first crucial stage of development of chemical screening assay using in vitro MS culture.

We investigated the potential of an assay wherein petioles of detached tomato leaves were placed in a solution (water or DMSO) and infested with adult psyllids. The feeding behavior of psyllids and efficiency of transmission of *Candidatus* Liberibacter psyllaurous were determined. At 7 days post infestation, several infested leaflet's edges were covered with eggs and continuous feeding by psyllid was observed. To determine

efficiency of transmission of CLps into leaflets, clean midribs were excised from leaves and petiole segments unexposed to psyllid feeding (petiole portion inside water) were used for testing CLps infection. Roots were also sampled from 13 leaves and 62% were detected positive for CLps infection. Figure 3.2A shows the percentage of detached leaves that were detected as CLps-infected by testing midribs and petiole segments in two replicate trials. The first trial had 6 infested detached leaves and the second replicate trial had 20 infested detached leaves. For each leaf, midribs and petiole segments from multiple leaflets were tested for CLps infection. The percentage of infected leaves detected by petiole segments was ranged from 80%-84% with an average of 6.01 X 10^9 CLps genomes per gram of fresh tissue in the two trials (Fig 3.2B). Consistency in low Ct values and high percentage of infected leaves was observed in both trials, indicating potential of this assay.

We used detached leaf assay to evaluate effects of chemical compounds effective in other pathosystem studies (Knoth et al. 2009). The chemical compound DCA was tested at two different concentrations (100 µM and 500 µM) with two tomato lines; susceptible variety Moneymaker and transgenic *CaBP22*-GUS line of tomato. The *CaBP22*-GUS transgenic line carries a *CaBP22* promoter fused to GUS reporter gene and is known to activate GUS expression in the presence of DCA (Baig, 2012). From each detached leaf, both petiole segments and leaflet midribs were tested at 14 dpi for CLps infection by real time quantitative PCR (qPCR). Average CLps genomes per gram of plant

tissue ranged from 1.14 X 10^9 to 3.56 X 10^8 and 1.64 X 10^9 - 3.29 X 10^8 in DCA treated and DMSO treated detached leaves respectively indicating no effect of DCA on CLps transmission or infection at these concentrations (Table 3.3).

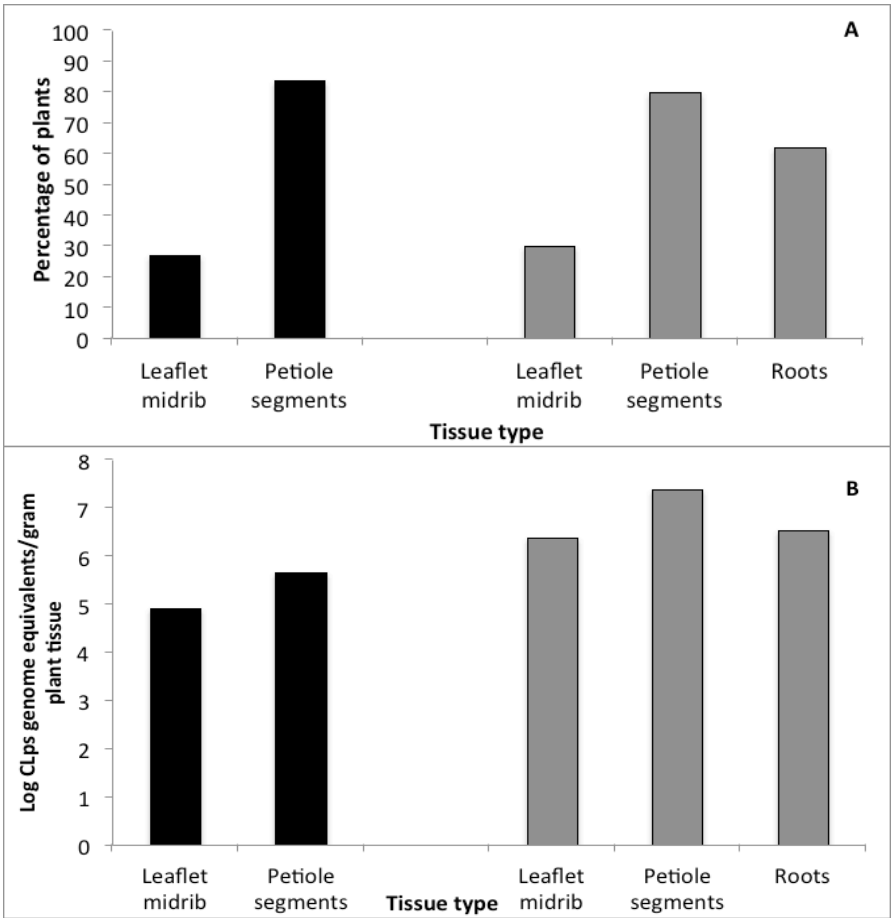

Figure 3.2: *Candidatus* Liberibacter psyllaurous infection in psyllid infested leaves of Tomato detached leaf assay. 3.2 A: The percentage of CLps-infected detached leaves among infested detached leaves detected by testing leaflet midribs or petiole segments (one leaflet midrib or one petiole segment for each detached leaf) and roots. 3.2 B: Log of CLps genomes present in different tissues of infected detached leaves. Legend: Trial 1 (black bars) and Trial 2 (grey bars).

Multiple leaves in this experiment exhibited phytotoxic effects most likely due to use of higher concentration of DCA than in previous studies (Knoth et al. 2009) and possibly as a result the RNA extracted from these leaf samples was of poor quality and was not

suitable to check GUS gene expression by RT-PCR. Consequently, chemical uptake by detached leaves could not be confirmed by checking GUS expression. An alternative approach to confirm chemical uptake via detached leaves was conducted. Detached leaves were incubated in a solution of water mixed with red color (Allura red (2-naphthalenesulfonic acid disodium salt), Ponceau 4R (Trisodium; (8Z)-7-oxo-8-[(4-sulfonatonaphthalen-1-yl)hydrazinylidene], and sodium chloride) and its uptake was confirmed by red color uptake along leaf veins and midribs (Fig 3.3). Although red dye uptake indicate chemical uptake by detached leaves, it is critical to confirm the small molecular weight compounds uptake by separate gene expression assay to validate the efficiency of this assay in high throughput chemical screening assays.

Table 3.2: List of chemical compounds

Chemical ID	Chemical description
DCA	3,5-Dichloroanthranilic acid
CMP442	2-(5-bromo-2-hydroxyphenyl)-Thiazolidine-4- carboxylic acid
CMP 199	Phenol, 2,4-dichloro-6-[[(3-methoxyphenyl)imino]methyl]-
BTH	1,2,3-Benzothiadiazole-7-carbothioic acid, S-methyl ester
CMP 815	3,5-Dichlorosalicylaldehyde
CMP 508	Benzoic acid, 3-fluoro-, 2-[(5-chloro-2 hydroxyphenyl) methylene] hydrazide
CMP 974	Benzenesulfonamide, 4-[[(3,5-dichloro-2-hydroxyphenyl) methylene] amino]-
INA	2,6-dichloroisonicotinic acid
DMSO	Dimethyl sulfoxide

Table 3.3: Effect of application of chemicals on CLps infection in CaBP22-GUS and Moneymaker tomato lines

Chemicals	Concentration	CLps genomes per gram of tissue	Number of CLps positive plants of the total infested plants
***CaBP22*-GUS**			
DCA	100 µM	1.11 X 10^9	3 of 3
DCA	500 µM	1.17 X 10^9	2 of 3
DMSO		1.64 X 10^9	1 of 3
Moneymaker			
DCA	100 µM	4.94 X 10^8	4 of 4
DCA	500 µM	2.19 X 10^8	4 of 4
DMSO		3.29 X 10^8	3 of 3

Figure 3.3: Red color uptake by detached tomato leaves.

Effects of foliar sprays of chemical compounds (25uM) on *Candidatus* Liberibacter psyllaurous infection in the *ein2-5* defense mutant.

In the defense mutant screening studies, the *ein2-5* mutant line had 53% of plants positive for CLps infection with an average of 43 CLps genomes per plant cell at 6 wpi, (Chapter 1). At 6 wpi, all CLps positive plants showed distinct disease related symptoms such as purple colored leaves, leaf curling, and excessive numbers of new young leaves. To determine the effect of defense-inducing chemical compounds on plant defense against CLps infection in *Arabidopsis*, the *ein2-5* plants were sprayed with six different chemicals and with three mixtures of three chemicals. For each chemical treatment, 8 plants were infested with psyllids and 2 plants were used as non-infested controls. The two objectives of this assay was to determine the effect of chemical compounds A) on plant recovery from CLps infection (Fig 3.4 white bars) and B) on transmission of CLps by psyllids into plants (Fig 3.4 grey bars). For objective A) plants were infested and then sprayed with chemical compounds at 4 wpi (post-infestation treatment, Fig 3.4 white bars) whereas for objective B) plants were sprayed first and then infested with psyllids 48 hours later (pre-infestation treatment, Fig 3.4 grey bars). The effect of chemical compounds was determined from phenotypic changes in plants in response to infection and from the estimates of the average genome numbers/plant cell. In objective A, to determine the effect of chemical compounds on plant recovery from CLps infection, we analyzed CLps positive plants treated with candidate chemical compounds BTH, CMP

199 and DCA along with control treatments (Water and DMSO). Except for DCA, all compounds resulted in some CLps positive plants (Fig 3.4A). The control treatment of DMSO had 5 of 8 infested plants positive for CLps. The average bacterial genomes/plant cell for control treatments of water and DMSO were 189 and 57 respectively, whereas for chemical compounds BTH and CMP 199 the number of bacterial genomes per plant cell was much lower, 11 and 5 respectively (Fig 3.4B). In objective B, to determine the effect of chemical compounds on CLps transmission into plants, all chemical treatments, except CMP442 resulted in some CLps positive plants. CMP815 had the highest number of plants (3 of 8 infested plants) positive for CLps infection but these infected plants had lowest bacterial genome numbers (10 CLps genomes /plant cell) in comparison to other chemical treatments (Fig 3.4 B). Interestingly, DMSO (control) treatment resulted in only 2 CLps positive plants with an average of 47 bacterial genomes per plant cell, which was lower than the numbers observed in the post-infestation chemical treatments. The CMP442 treatment did not result in any CLps positive plants and therefore was considered as a compound that needed further validation to determine its effect on CLps transmission.

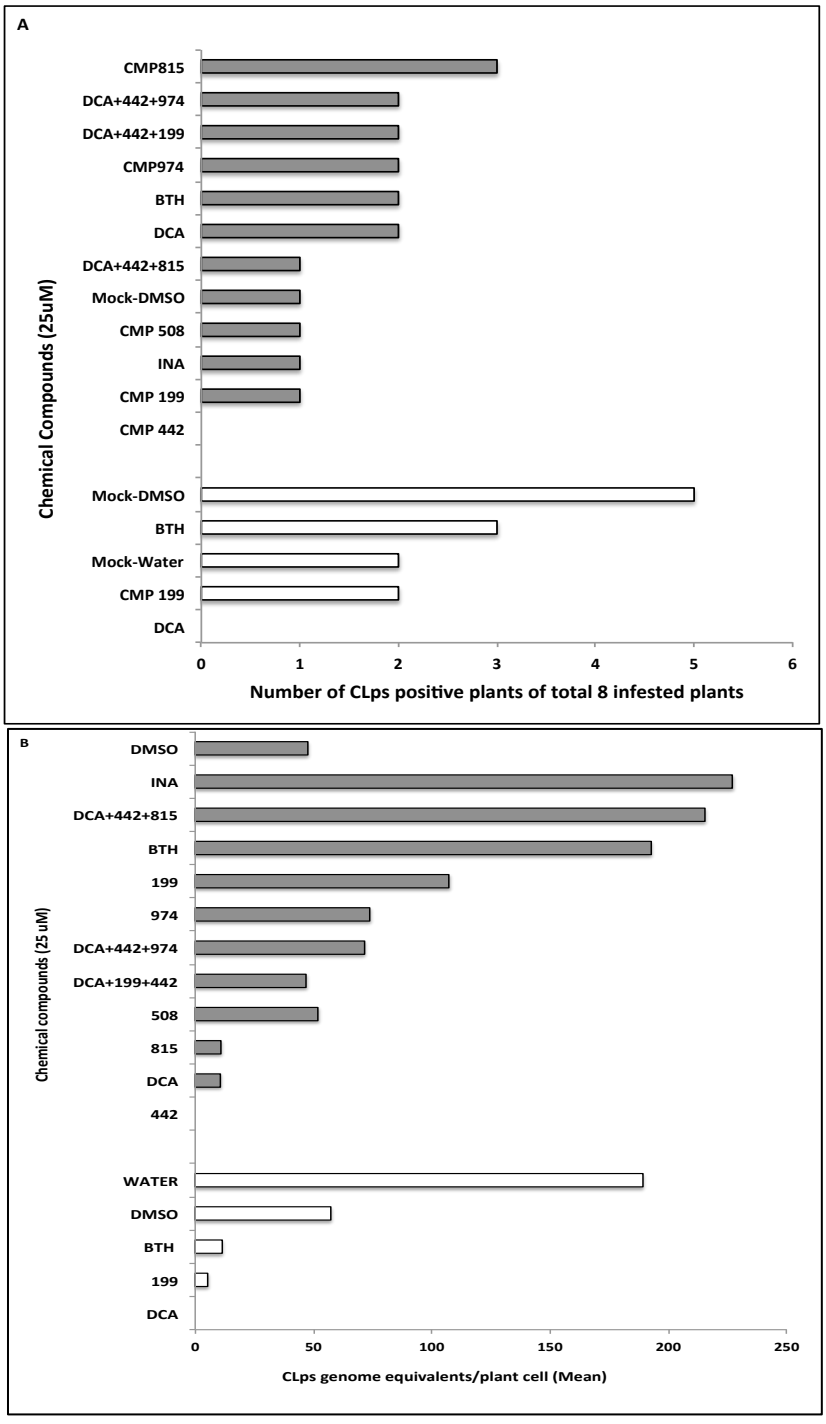

Figure 3.4: Effect of foliar spray of chemicals on *Candidatus* Liberibacter psyllaurous infection. 3.4A: Number of *Arabidopsis ein2-5* plants detected positive for CLps infection in pre-infestation (grey bars) and post-infestation (white bars) chemical treatment, 3.4B: CLps genome equivalents /plant cell following pre-infestation (white bars) and post-infestation (grey bars) chemical treatment.

To study the effect of chemical compounds on phenotype change specifically in leaf numbers, a comparative statistical analysis of control and chemical treatments was performed with one-way ANOVA and t-test. Rosette leaves of all experimental plants were counted on plants at 7 wpi. The statistical analysis (one-way ANOVA, $p>0.05$) indicates no significant effect of chemical compounds on leaf numbers in CLps positive plants in pre-infestation group of plants, when chemical compound treated CLps-infected plants were compared along with DMSO treated CLps-infected plants (Fig 3.5B). In the post-infestation treatment, the number of leaves on infected plants was consistently lower in comparison to non-infected plants for both control and chemical treatments (Fig 3.5A). In addition, there was no significant difference in leaf numbers of CLps positive plants of chemical treated and DMSO (control) treated plants (Student's t-test, $p>0.05$). The number of plants showing discoloration of leaves was analyzed for all treatments to study the effect of chemical compounds on CLps infection associated symptom. Interestingly, with chemical treatments in the pre-infestation trial, several plants treated with chemicals and negative for CLps showed discoloration. All CMP815 treated plants (both infected and non-infected) showed discoloration (Fig 3.6). In post-infestation, only infected plants showed discoloration and no non-infected plant displayed discoloration. Lack of significant difference in the number of discolored CLps positive plants between chemical and DMSO treatments indicates no effect of chemical compound on symptom development (Fig 3.6, one-way ANOVA).

Lack of significant difference in symptomology between chemical treatments and control treatment and between CLps positives and CLps negative plants for both objectives indicates that compounds were ineffective in suppressing symptoms.

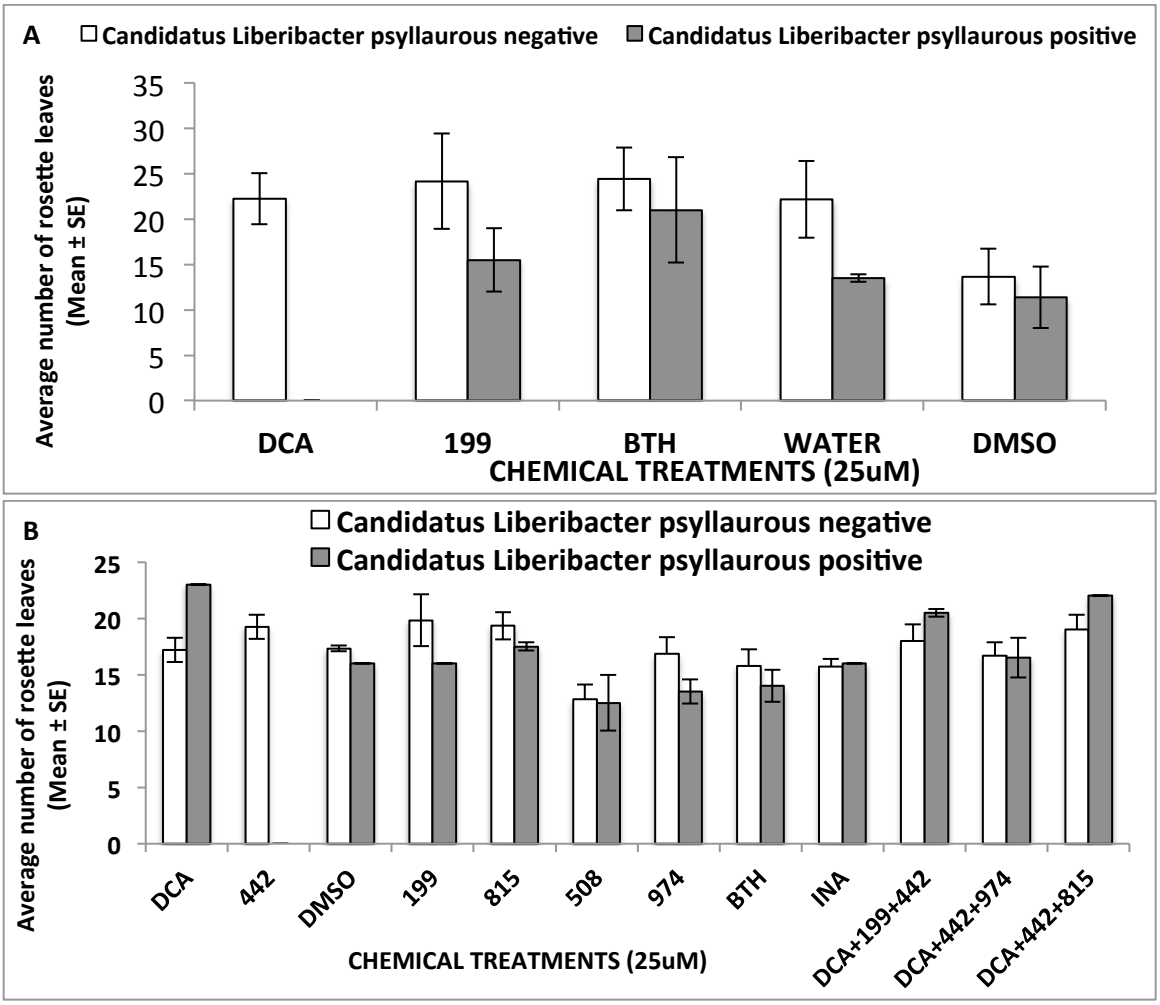

Figure 3.5: Number of rosette leaves on chemical treated plants with pre and post infestation treatments at 7 wpi. A) Average number of rosette leaves on infested plants treated with chemicals at 4 wpi (Post-infestation) B) Average number of rosette leaves on plants infested 48hrs post chemical treatment (Pre-infestation). White bars- CLps negative plants, Grey bars- CLps positive plants. Data represented in A and B is a mean (± standard errors) of rosette leaves of all the replicates for CLps positive and CLps negative plants in the respective treatments. No significant difference was detected on the rosette leaf numbers of CLps positive plants when treated with chemicals for both pre-infestation ($p>0.05$ one way ANOVA) and post-infestation experiments ($p>0.05$ Student's t-test).

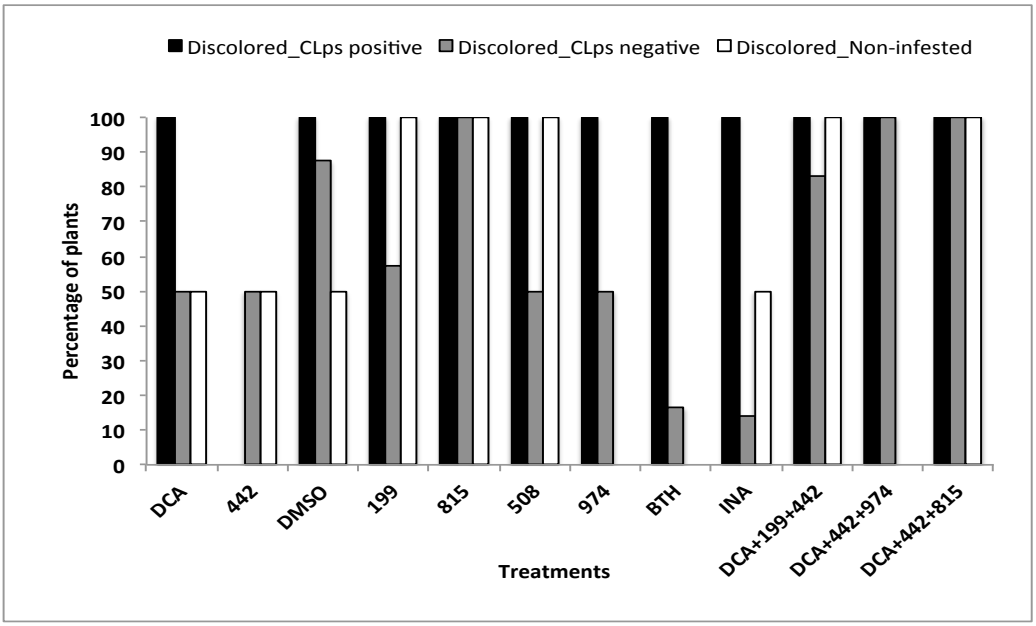

Figure 3.6: Percentage of discolored plants in chemical and control treatments in the pre-infestation experiment. The number of discolored CLps-positive plants was not significantly different in the chemical and control treatments (one-way ANOVA, $p>0.05$).

Validation of effects of chemical compounds 442 and DCA on the plant responses to *Candidatus* Liberibacter psyllaurous infection

In preliminary screening, chemical compound 442 treatments did not result in any CLps positive plants when 8 plants were sprayed with chemical and then infested with psyllids. To validate its effectiveness in inducing plant defense to CLps transmission after chemical treatment, CMP442 was tested with 25 replicates. At 4 weeks, 25 *Arabidopsis* plants were sprayed with 25 µM CMP442 and 25 plants were sprayed with the control treatment (0.5% DMSO). Of the 25 replicates, 20 were infested with psyllids 48hrs post chemical treatment and 5 were left as non-infested controls for both CMP442 and

DMSO treatments. At 8 wpi, 2 of 20 and 3 of 20 infested plants were detected positive for CLps infection in CMP442 and DMSO treatments respectively. The average bacterial genome numbers / plant cell was higher in CMP442 treated plants in comparison to control DMSO treatment (Fig 3.7), however this difference was not statistically significant (Welch t-test, $p>0.05$). A different validation experiment was conducted to determine the potential of chemical compounds DCA and 442 for their effects on plant recovery from CLps infection. At 4 wpi, prior to symptom development, chemical compounds 25 µM DCA, 25 µM 442 and 0.5% DMSO (control treatment) were applied on 6 replicates detected positive for CLps at 4 wpi (Average of 0.35 bacterial genomes/plant cell, figure 3.8A). Four weeks post chemical treatment, analysis of bacterial genome numbers in plants showed that control DMSO treatment had higher bacterial cell numbers in comparison to CMP442 and DCA treatments in CLps positive plants (Fig 3.8B), but was not significantly different (Student's t-test, $p>0.05$). Phenotype analysis (Fig 3.9A-D) of these CLps positive plants treated with chemical and DMSO treatments revealed no significant effect of chemical treatments on the symptoms such as leaf curling, leaf color changes, rosette leaf and axillary leaf numbers in infected plants of control plants and chemical compound treatments.

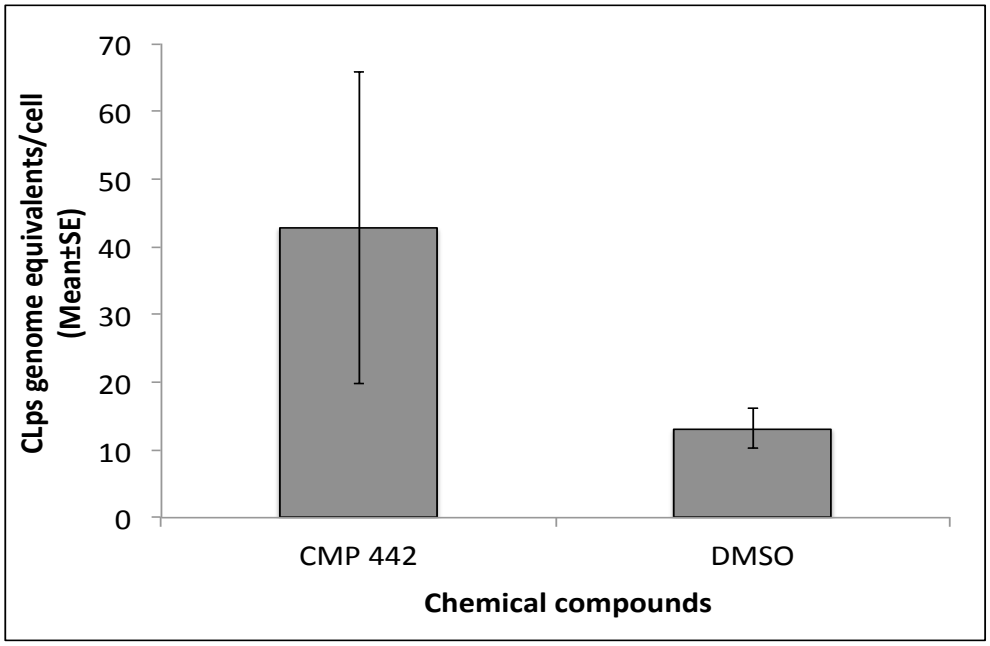

Figure 3.7: Pre-infestation validation screen. CLps genome equivalents / plant cells in CMP442 and DMSO treated plants (detected positive for CLps in validation screen. Data represented is mean (± standard errors) of CLps genome equivalents /plant cell. No significant difference was detected between DMSO and CMP442 treatments (Welch t-test $p>0.05$).

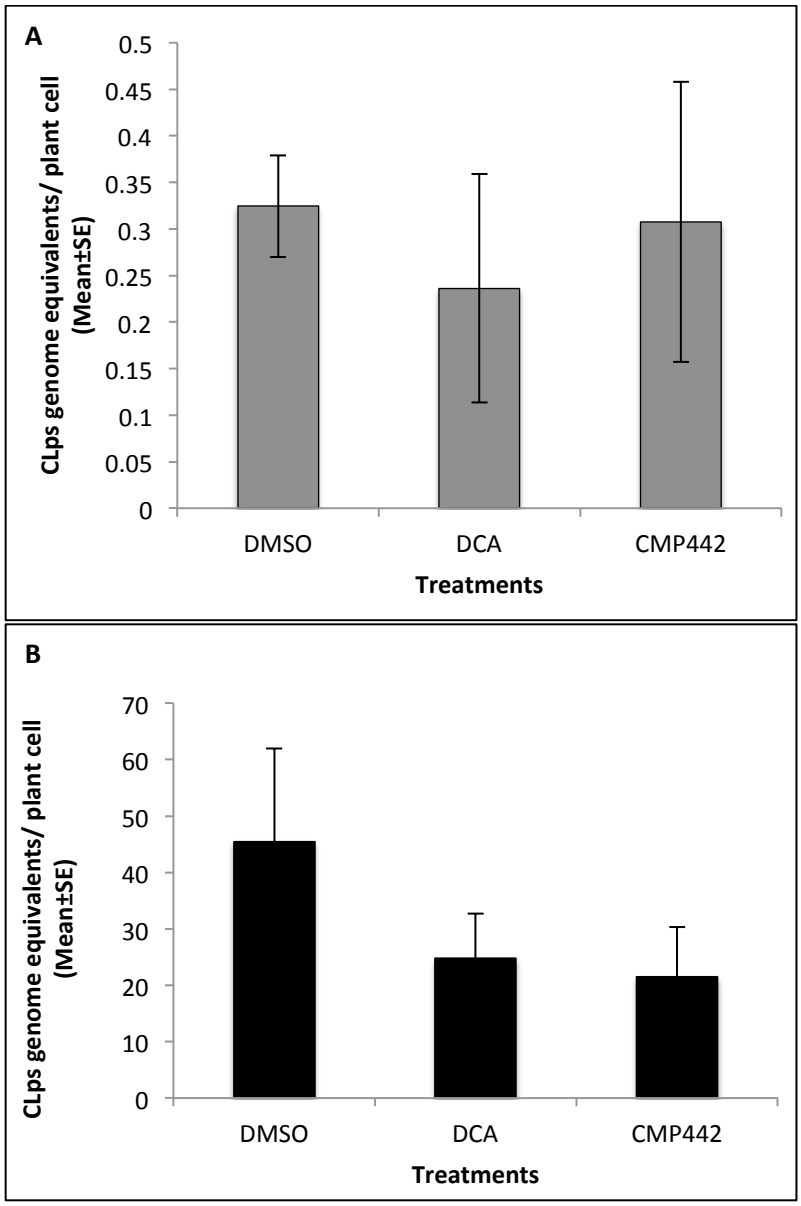

Figure 3.8: Effect of chemical compounds on bacterial growth in plants of post infestation validation screen. Graphical representations of average number (with standard error bars) of CLps genome equivalents/plant cell at 4 wpi (grey bars) and at 8 wpi (black bars, after chemical treatment). No significant difference was detected in the number of CLps genome equivalents/plant cell between DMSO and chemical treatments (Student's t-test, $p>0.05$).

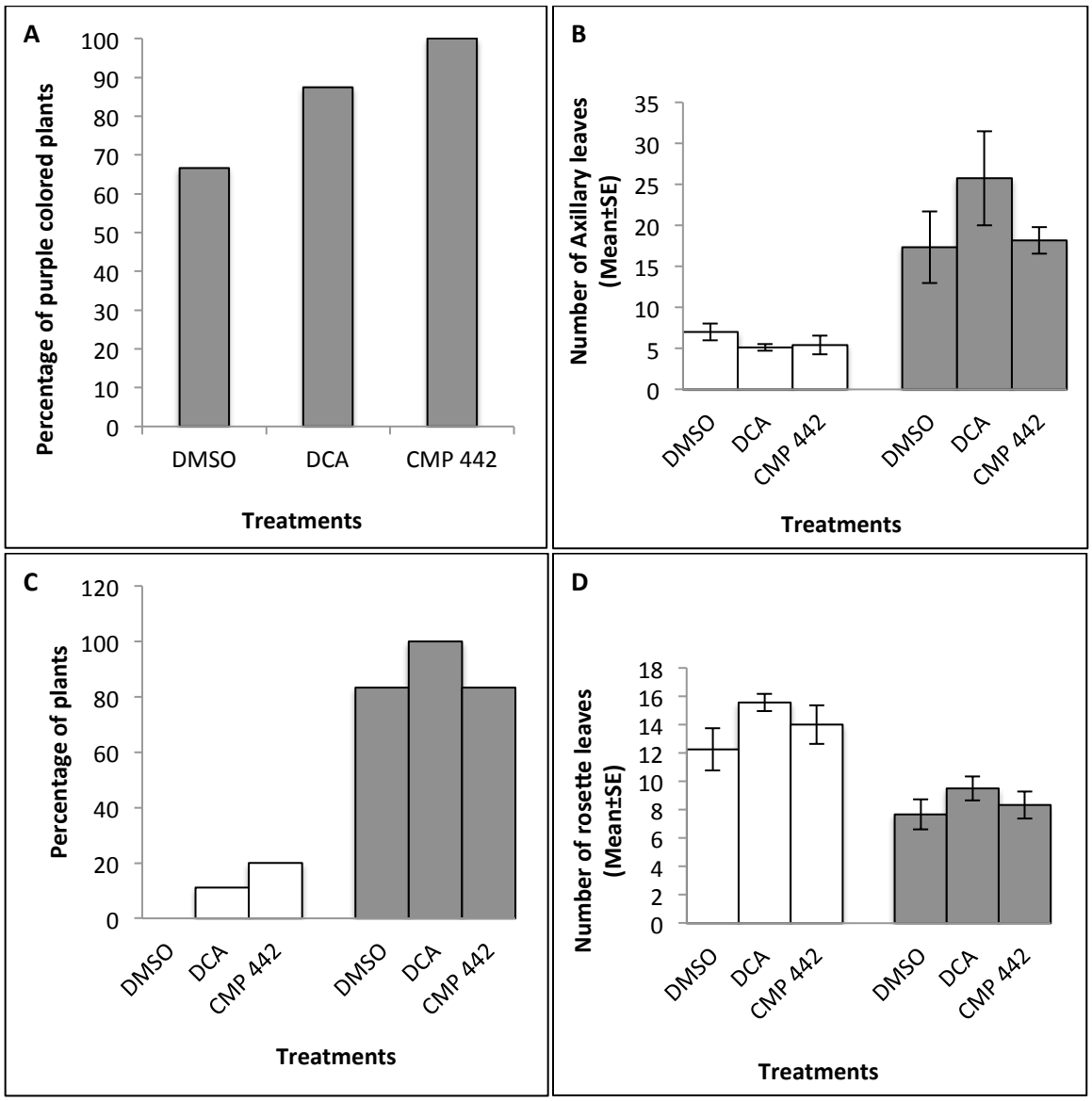

Figure 3.9 (A-D): Analysis of symptoms in post-infestation validation screen. Analysis of symptoms at 7 wpi of non-infected (white bars) and infected *Arabidopsis* plants (grey bars) treated with 0.5% DMSO, 25 µM DCA and 25 µM CMP442. **A)** Percentage of infected plants showing discoloration (no non-infected plants showed discoloration). **B)** Average number (± standard errors) of axillary leaves in non-infected and infected plants**. C)** Percentage of non-infected and infected plants showing leaf curling. **D)** Average number (± standard errors) of rosette leaf numbers in non-infected and infected plants. For A and C, no statistical significance was detected in the percentage of CLps-positive plants displaying discoloration for chemical and control treatments (Chi Square test, $p>0.05$). For B and D, no statistical significance was detected in the number of rosette leaves of CLps-positive plants for chemical and control treatments (Student's t-test, $p>0.05$

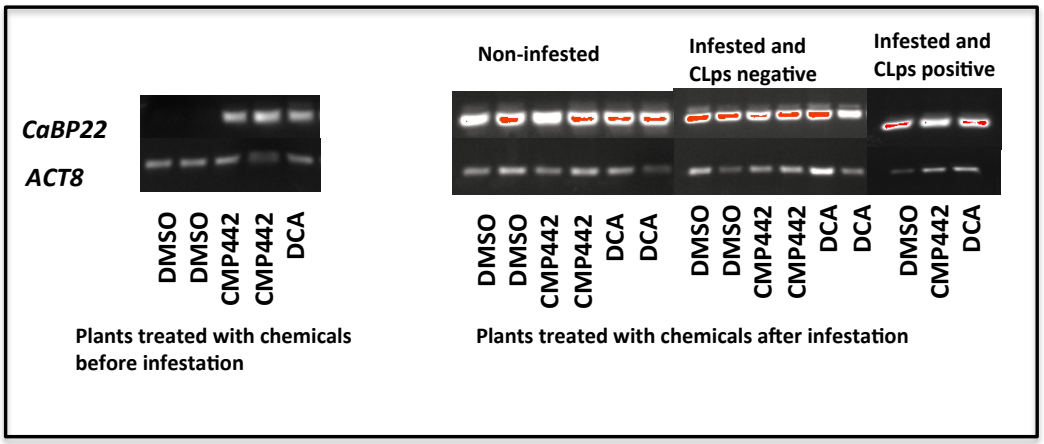

Figure 3.10: RT-PCR results showing transcript levels of *CaBP22* and *ACT 8* (loading control) to confirm chemical uptake by foliar sprays in both objectives. (Left)- Plants were treated with chemicals and then infested with psyllids (Right) – Plants were infested with psyllids for CLps infection and then sprayed with chemicals/control treatments.

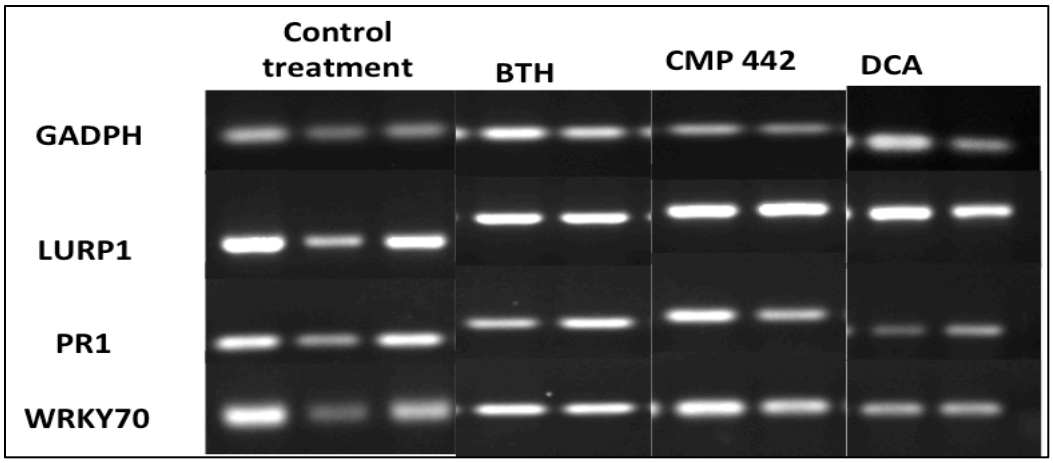

Figure 3.11: Gene expression in leaves of sweet orange seedlings treated with three defense-inducing chemicals. RT-PCR results of housekeeping control gene *GAPDH*, and defense genes *PR1, LURP1* and *WRKY70* 18 hours after spraying with DCA, BTH, CMP442, and control (DMSO). PCR products of three control seedlings and two seedlings from each chemical treatment after 30 cycles were electrophoresed on 2% agarose gel).

Induction of *CaBP22* gene was detected in chemical treated plants as well as in plants from which tissues were collected prior to chemical treatment.

To confirm the chemical uptake in CMP442 and DCA treated plants by foliar-spray technique, expression levels of the *CaBP22* transcript were examined along with *ACTIN8* as a loading control (Fig 3.10). In plants treated with chemicals before infestation with psyllids, transcript levels of *CaBP22* gene were higher in DCA and CMP442 treated plants in comparison to control 0.5% DMSO treated plants (Fig 3.10 left). For plants sprayed with chemicals after infestation, (Fig 3.10, right), chemical uptake by foliar sprays could not be confirmed due to lack of difference in transcript levels in all three treatments (non-infested plants, plants infested with CLps positive psyllids and detected negative for CLps, plants infested with CLps positive psyllids and detected positive for CLps). All treatments including DMSO, showed significant up regulation of *CaBP22* transcript in comparison to plants treated after infestation (Fig 3.10, left). Wounding has been shown to be associated with changes in *CaBP22* expression changes (Knoth et al. 2009). It is very likely that up-regulated expression of *CaBP22* transcripts in DMSO and other chemical treatments could result from injury caused by tissue collection (for detecting CLps positive plants) prior to chemical treatment.

Foliar spray of chemical compounds that promote innate immunity in *Arabidopsis* on Sweet Orange (*Citrus sinensis*) seedlings results in induction *LURP1*, *WRKY70* and *PR1* relative to housekeeping gene GADPH in chemical treated and control (surfactant/DMSO) treated plants, but no difference in transcript levels was observed between chemical treatment and mock treatment.

In *Arabidopsis thaliana,* chemical compounds 5 mM DCA, 5 mM 442 and 5 mM BTH treatment induced *WRKY70* and *LURP1* genes within 48 hours of chemical treatment (Knoth et al., 2009; Rodriguez-Salus, 2012). To determine if similar changes are induced in sweet orange seedlings when sprayed with DCA and CMP442; at 18hrs post chemical treatment, leaves at similar stages of development were tested for expression of three defense-inducing genes, *LURP1, WRKY 70* and *PR1*. RT-PCR analysis showed that these genes were expressed in all treatments including control treatment (Surfactant/DMSO) at similar levels (Fig 3.11). Due to lack of differential regulation of chemical induced genes in citrus, this study was not able to confirm if the chemicals BTH, CMP442 and DCA induced changes similar in sweet orange seedlings like *Arabidopsis thaliana*. These genes were up-regulated in comparison to housekeeping genes thereby indicating there was some triggering mechanism, but if this triggering was induction by surfactant (Widespread max) or injury caused during transport of plants from green house to laboratory needs to be determined.

DISCUSSION:

The core objective of this study was to identify small chemical compounds that can elicit plant defenses against *Candidatus* Liberibacter psyllaurous and use these in development of control strategies for HLB disease of citrus. *Arabidopsis thaliana* has been successfully used in studies wherein chemical compounds were used to understand biological processes and defense responses. Knoth's (Knoth et al. 2009) and Rodriguez's studies (Rodriguez-Salus 2012) provide crucial evidence of successful use of *Arabidopsis thaliana* in identification of plant defense elicitors. In my study, we investigate the potential of this well studied, resourceful and tractable plant species in conducting chemical screening with "Psyllid Yellows" pathosystem. To develop a chemical screening approach to identify defense-eliciting compounds, it was necessary to consistently generate a high number of infected plants. We investigated different assays such as in vitro and hydroponic culture based assays to develop high throughput chemical screening assays but these did not result in a consistent and high percentage of CLps infected *Arabidopsis* plants. A detached leaf assay based on a citrus detached leaf assay (Ammar et al., 2013) was investigated with *Solanum lycorpersicon* var. Moneymakar and psyllid yellows system. Petioles of leaves with 3- 4 leaflets from plants grown in an insect free environment were incubated with CLps-infected psyllids to determine CLps transmission by psyllids. 80-84% of leaves were detected positive for CLps infection, which was higher than other assays. This was the only assay tested that

showed high CLps infection percentage with leaf samples and has potential to be developed into chemical screening. However, in this detached leaf assay, chemical uptake couldn't be confirmed by gene expression analysis due to poor quality RNA but a different dye experiment confirmed the movement of dye chemicals from water into leaves. The chemical compounds used in screening are small molecular weight compounds; therefore their ability to diffuse through cell membranes in detached leaves needs further verification by molecular assays.

Another chemical treatment technique known as foliar spray was investigated with *Arabidopsis thaliana*-CLps-psyllid pathosystem. This technique was used to expose soil grown plants to small molecular weight chemical compounds at a concentration found effective in another plant pathosystem. Two screens (preliminary and validation) were conducted to determine chemical effects on CLps infection in *Arabidopsis thaliana*. Chemical effects on plants defense response were determined by analyzing symptoms and bacterial genomes/ plant cell. In the preliminary screen, single and combination of 3 chemicals were tested at a total concentration of 25 µM. Observations indicated no phytotoxic effect of these compounds on *Arabidopsis* plants. Two chemical treatments DCA and CMP442 applied before and after psyllid infestation yielded promising results. Either no plant was detected positive or low bacterial titer was found in these chemical treatments. Many of these screening studies are pursued in a high throughput manner wherein hundreds or thousands of chemicals are tested simultaneously to identify

compound inducing desired effects (Armstrong et al., 2004; Schreiber et al., 2008; Serrano et al., 2007). In our study, it was difficult to develop the widely used multi-well plate-screening assay because plants can only be infected by psyllids and must be large enough for infestation; therefore we explored the possibility of testing combinations of chemical compounds. While testing a combination of chemical compounds, one must consider that the combined chemicals can function independently, antagonistically, synergistically or additively. When there is antagonistic activity, one compound would reduce the effect of another. In case of synergistic activity, compounds can function together at their minimal active concentration and have increased effect on system in comparison to individual effect produced by independent compounds. In case of additive activity, the effect of combination of chemicals is observed when compounds are applied at concentration equal to the sum of minimal active concentration. CMP199 has been reported to be more potent than DCA at lower active concentration and it has been indicated to work additively with CMP442 (Rodriguez-Salus, 2012). Compounds DCA and 442 were also tested in combination with CMP199, CMP815 and CMP974 to determine their combined effect on CLps infection. However, no significant effect on bacterial titer was observed when CMP199, CMP442 and DCA were applied in combination of 25 µM concentration indicating lack of additive or synergistic effect on triggering plant defenses to CLps infection at this concentration. This study can be further investigated by testing different concentration of combined chemicals.

In the validation screen, CMP442 treatment 48hrs prior to infestation resulted in 2 CLps positive plants of 20 infested plants. Although the number of CLps positive plants was low for both DMSO (3 plants) and CMP 442 (2 plants), bacterial titer was higher in CMP442 treated plants. Statistical analysis of bacterial genomes/plants cell showed that CMP442 treated plant bacterial titer was not significantly different for plants treated with CMP442 than DMSO. Phenotype analysis of all CLps positive plants of CMP442 and DMSO treatments (Figure 3.12) showed similar intensity of disease related symptoms such as discoloration and excessive small leaf growth. Based on these results it was evident that CMP442 had no effect on CLps transmission into *Arabidopsis* plants at the 25 µM concentration. However, these results are based on phenotype and bacterial cell values of 2-3 plants detected positive for CLps infection, and needs further validation with more replicates to confirm chemical compound effects. It could be interesting to determine the effect of different higher concentrations of CMP442 on CLps transmission by psyllids.

Figure 3.12: CMP442 and DMSO treated and CLps positive plants.

Based on gene expression analysis of CLps infected *Arabidopsis* (Chapter 2), it is evident that several genes involved in SA defense pathway are activated. Studies suggest the potential mode of action of CMP442 and DCA in SA pathway; therefore it was interesting to observe that DCA and CMP442 treated plants showed less bacterial growth in comparison to control DMSO treatment in the validation experiment. Although, the chemical treated plants showed less bacterial growth (Fig 3.8B) in comparison to control (DMSO) plants, lack of statistical significance makes DCA and CMP442 treatments ineffective in triggering adequate plant responses to CLps infection. Before accepting lack of effectiveness of any chemical compounds, several factors needs to be considered with psyllid yellows when screening defense eliciting compounds, A) timing of chemical application and corresponding stage of infection, B) growth stage of plant, and C) concentration of chemical compound required to elicit adequate response.

In this study, plants were sprayed with 25 uM solutions of each chemical compound or with a 25 uM combination of three chemical compounds. This concentration may not be effective in triggering adequate responses at that stage of infection. Especially for combination of chemicals, the synergistic or additive effects of chemicals should be considered and concentration needs to be adjusted accordingly. The literature shows that research on chemical screening assays with a biological system that involves vector transmission of bacteria to a host that is resistant to vector but not to the bacterium has been very limited. No published study was found wherein the effects of screened small molecular weight chemicals were studied on any such system thereby restricting my ability to relate my results with other systems. However, despite these challenges, I believe my study on examining chemical screening assays could help researchers in future on various aspects of assay development for screening small molecular weight chemicals with such pathosystem.

CLps positive plants were treated with chemical compounds when no symptoms were visible on plants (4 wpi). The phenotype of symptomatic plants was analyzed to determine differences between infected plants treated with DCA, CMP442 and DMSO. There were no significant differences between infected plants sprayed with the control and chemicals in rosette leaf numbers, leaf curling, discoloration and excessive leaf growth. For plants treated with chemicals after infestation, phenotype analysis indicated that discoloration of leaves and fewer rosette leaves are observed in plants

detected positive for CLps infection. The slower growth of infected plants regardless of post infestation chemical treatments indicated no effect of chemicals on symptom development. To confirm if the foliar spray technique was effective for chemical application to the plants, leaf tissues or entire *Arabidopsis* seedlings were collected to analyze expression of *CaBP22*, a defense gene induced in response to DCA and CMP442 in other studies (Knoth et al. 2009; Rodriguez-Salus 2012). RT-PCR results indicated that *CaBP22* was induced only in chemical treated plants and not in DMSO treated plants when plants were sprayed with chemicals before infestation. However, plants that were sampled to detect CLps infection showed *CaBP22* induction, irrespective of infestation and chemical treatments. These results show that *CaBP22* can be induced in response to chemical treatment and wounding. To confirm chemical uptake in plants from which leaf samples were collected prior to treatment, *CaBP22* gene is not an ideal reporter gene. In these experiments, it was crucial to identify CLps infected *Arabidopsis* before chemical treatment because of low and variable rate of infection. Transmission of CLps by psyllids into plants has been bottleneck in using *Arabidopsis*/CLps as model plant-pathosystem, most likely resulting from *Arabidopsis*'s resistance to *Bactericera cockerelli*. The percentage of CLps positive psyllids ranged from 92-98%, but the percentage of CLps positive *Arabidopsis* plant was always than less 50%, which is inadequate to confirm the magnitude of chemical effects statistically. Testing for CLps can be avoided if a consistent high percentage of CLps positive *Arabidopsis* plants are obtained post infestation. This could be possible by exposing plants with more than 10

psyllids. We observed that psyllid were able to transmit CLps into *coi-1* plants with better efficiency, and its suitability in such chemical screening assays could be further investigated.

The effect of three chemicals DCA, CMP 442 and BTH applied as foliar sprays to 3 months old *Citrus sinensis* seedlings was also investigated. The objective was to verify if these chemicals induce responses similar to *Arabidopsis thaliana*. Most of these chemical compounds have a transient effect and therefore at 18hrs post chemical treatment leaf samples from control (DMSO) and chemical treated plants were tested for chemical uptake by gene expression analysis. RT-PCR analysis of *PR1, LURP1* and *WRKY70* transcript levels indicate no difference in chemical and control treatments. These results were inconclusive for confirming chemical uptake in citrus.

CONCLUSION:

This study shows the potential of foliar spray technique and detached leaf assay in conducting chemical screening with *Arabidopsis* and Tomato. The chemical screening results with *Arabidopsis*/CLps demonstrate that candidate chemicals can be tested individually and in mixtures by foliar sprays. In an effort to develop high-throughput chemical screening, potential of application of a mixture of chemical compounds was investigated. Upon identification of potential chemical compounds, this study can be pursued further in two directions. One direction could lead towards identification of the chemical's targets in plants and develop a transgenic citrus that would be HLB resistant and the second direction could be to use these novel defense-inducing chemical compounds as alternatives to pesticides.

GENERAL CONCLUSION

Understanding plant-pathogen interactions is crucial for the development of control strategies for diseases, especially when these diseases cause significant damage to food or fruit crops. Huanglongbing is one such disease that has been prevalent for a century and has spread in several citrus growing areas around world. In recent years, HLB has spread in almost all citrus growing counties in Florida (Manjunath et al., 2008) and threatens California citrus industry. The available measures such as nutrient supplementation and frequent foliar sprays of pesticides for vector control have not been completely effective in controlling vector or pathogen and have increased the production and maintenance costs of citrus significantly (Gottwald et al., 2012). Several inherent challenges associated with Citrus/HLB system such as lack of in-vitro cultures of HLB pathogen, longer generation time of citrus, incompatibility problems, few genomic resources and regulatory issues limit the progress in understanding of this disease. This study demonstrates the potential of a model plant-pathogen system to aid Citrus-Huanglongbing research and tests methods to screen defense inducing small molecular weight chemicals that can be further explored in designing disease control strategies. Using a model plant-pathosystem can rapidly provide critical information about plant's defense mechanisms against a pathogen. *Candidatus* Liberibacter psyllaurous (CLps) is associated with a solanaceous disease "Psyllid yellows" (Hansen et al., 2008) and shows several similarities with HLB pathogen. Similarities in the mechanism of pathogen

transmission, *16SrDNA* relatedness and symptomology indicate potential of CLps as a model pathogen to study interactions of *Candidatus* Liberibacter species associated with HLB disease. *Arabidopsis thaliana* has been shown to respond to various pathogens in similar manner to their natural hosts and has provided useful information that can aid in disease research (Makandar et al., 2006; Sandhu et al., 2009). Several reports demonstrate its potential in understanding responses to citrus pathogens or understanding gene functions in various diseases (Lu et al., 2013; Zhang et al., 2010). Herein, I show that *Arabidopsis* can be infected with *Candidatus* Liberibacter psyllaurous by psyllid transmission. Phenotypic and transcriptional responses of *Arabidopsis* in response to CLps infection were analyzed in detail to aid HLB research by providing a plant-pathosystem that has numerous genetic and genomics resources.

As in any new plant-pathosystem, it was crucial to establish an optimum method for pathogen transmission into plants. *Arabidopsis* responses to psyllid transmission of CLps were examined in a few small-scale experiments. In these experiments, we found that *Bactericera cockerelli* transmits CLps into two widely used ecotypes, Col-0 and L*er*-1. By 3 wpi, systemic CLps infection was confirmed in multiple plants, however no distinct symptoms related to CLps infection was visible on any infected plant. Interestingly, no effect of bacterial infection on plant growth was observed at 3 wpi. In addition to Col-0 and L*er*-1, seventeen genetically diverse *Arabidopsis* ecotypes were tested for CLps infection and all ecotypes were detected positive, albeit with variable bacterial titers

and variable proportions of infected plants. A repeat experiment with more replicates failed to confirm the differences among ecotypes due to highly inconsistent results. Despite these inconsistencies, it is evident that CLps can infect *Arabidopsis thaliana* by psyllid transmission and can systemically spread in plants. These experiments were concluded at 5 wpi and no plant showed any visible changes in phenotype despite high bacterial cell numbers. To determine if *Arabidopsis* is tolerant to CLps and to genetically dissect the basis for tolerance, 5 defense-compromised lines were examined along with wild type lines for an extended time (6 wpi-7 wpi). Except *coi-1*, all defense-compromised and wild type lines were detected positive by 3 wpi and increased bacterial titer was evident by 6 wpi. Among all lines, *NahG*, *ein2-5*, and Col-0 had a higher percentage of CLps positive plants, higher titer of CLps, and also displayed distinct symptoms such as discoloration, overgrowth of axillary leaves, leaf curling and slower growth. A subsequent detailed study of phenotypic and transcriptional changes in response to CLps infection was conducted with *Arabidopsis NahG* and Col-0 lines. A significant difference in the phenotype of CLps infected plants was observed in comparison to non-infested and non-infected plants. Plants were significantly delayed in growth, which was evident by fewer rosette leaves and slower stem development, especially in Col-0. The notable symptoms of CLps infected Arabidopsis (*NahG* and Col-0) were excessive growth of small size axillary leaves, discoloration and leaf curling. Based on these symptoms, it is clearly evident that CLps caused significant alteration in plant development. However, most of these symptoms became distinct at 8 wpi and not at

early infection stage indicating latency in CLps related responses. Transcriptional analysis of CLps positive and symptomatic plants indicates several similarities with gene expression of Citrus/HLB system. Several genes encoding pathogenesis related protein, WRKY transcription factors, and enzymes involved in carbohydrate metabolism were expressed in similar patterns to those reported in Citrus/HLB. In addition to these genes, β *-1,3-GLUCANASE* gene encoding callose degrading enzyme was significantly up regulated in *Arabidopsis*. Several genes involved developmental processes were differentially expressed in *Arabidopsis* and these are not yet reported in HLB. These genes encode MADS box transcription factors that are involved in floral development. Transcription factors such as AGL42, AGL8 and AGL3 involved in floral transition were significantly down-regulated in response to CLps infection. Among notable DEGs in response to CLps infection, several genes were up-regulated in response to oxidative stress. Genes encoding enzymes such as peroxidases, superoxide dismutase and Glutathione-S-transferase were up-regulated, indicating triggering of basal defense in plants. Overall, transcriptional analysis of *Arabidopsis* revealed that the plant triggers defense related responses to CLps infection, but these may not be adequate and as the result plants develop severe symptoms by 8 wpi. Although the overall phenotype analysis did not show significant differences in plant symptoms between Col-0 and *NahG* lines, visual observations indicated that Col-0 plants showed more severe symptoms than *NahG*. In Col-0, almost all infected plants were completely discolored in comparison to presence of few discolored leaves on infected *NahG* plants. Evidence of

absence of stems on several CLps positive Col-0 plants as opposed to presence of stems in all *NahG* CLps positive plants, was also an indication of slower growth of Col-0 infected plants in comparison to *NahG* infected plants. These responses need further investigation and validation to determine potential tolerance in *NahG* line.

Lastly, I examined two assays to determine their potential application in chemical screening; a tomato detached leaf assay and foliar sprays of chemicals (Chapter 3). In the tomato detached leaf assay, a consistently high percentage (80-84%) of CLps positive replicates were evident in multiple experiments. Although chemical uptake was confirmed by dye experiment, this assay requires further validation of chemical uptake by gene expression analysis. If chemical uptake is confirmed by any technique, this assay has a strong potential for screening chemicals both singly and in multiplex. I also tested foliar spray application of chemicals with soil grown *Arabidopsis* plants to determine its potential with *Arabidopsis*-Psyllid-CLps system. Different candidate chemical compounds found effective in other defense studies were tested individually and in combinations. No compound showed significant effect on bacterial infection in replicate experiments. Chemical uptake by plants when foliar sprays were used was confirmed by expression analysis of gene *CaBP22* (a reporter gene induced in response to candidate chemicals). Generating a consistent number of CLps positive plants has been the bottleneck of development of these assays. Since, most *Arabidopsis* /CLps assays are dependent on the CLps transmission efficiency of psyllids, it is necessary to explore

more lines suitable for psyllid feeding or possibly expose the currently researched lines to a high number of psyllids to produce high percentage of CLps positive plants.

Based on all the results in this study, *Arabidopsis* was found susceptible to *Candidatus* Liberibacter psyllaurous, a close relative of HLB pathogen. The development of CLps related symptoms and increase in bacterial titer over time provides evidence of CLps transmission by psyllid and susceptibility of *Arabidopsis* to Liberibacter. I conclude that *Arabidopsis thaliana* shows distinct basal defense responses to CLps infection. The R-gene mediated resistance may not be evolved in *Arabidopsis* or may be insufficient for complete resistance. The lack of psyllid survival on any *Arabidopsis thaliana* lines shows non-host response to *Bactericera cockerelli* psyllid and hence the lack of natural exposure to Liberibacter pathogen transmitted by psyllid. So, it is possible that co-evolution of R-genes to Liberibacter effectors is still lacking. This offers an opportunity to utilize numerous functional resources and extensive knowledge of Arabidopsis to manipulate such defense to create Liberibacter resistant species. Use of defense mutant lines (single or double) that are compromised in either one or multiple defense pathways and the systematic dissection of their effects can aid in identifying genetic components that play crucial role in Liberibacter defenses. Such studies can offer valuable insight into plant defenses at basal as well as R-gene resistance levels.

The core objective of this study was to understand the *Arabidopsis* responses to Liberibacter and determine similarities with Citrus /HLB. This studies shows distinct similarities between *Arabidopsis*-CLps responses and Citrus-HLB responses and can be potentially used as resource for HLB related research. The *Arabidopsis*-CLps pathosystem offers a reliable and faster detection of Liberibacter, which is often not possible with citrus/HLB system in experiments. Due to lack of uniform distribution of bacteria and longer period of symptoms development, it is difficult to determine the spread of pathogen and eventually confirm effect of any chemicals, genetic manipulation etc. with citrus species. With *Arabidopsis*-CLps pathosystem, an entire plant can be easily harvested and tested for Liberibacter. Such testing of Liberibacter and the effect of any desired manipulations/application can be determined as early as 9 weeks (or 6 wpi) of total experimental duration. In addition to the significant advantage of time, availability of numerous genetic and genomics tools and resources makes *Arabidopsis*/CLps a potential model plant-pathosystem for HLB research.

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