Salicylic acid mediated immune response of *Citrus Sinensis* to varying frequencies of herbivory and pathogen inoculation

Freddy Ibanez^{1*}, Joon Hyuk Suh², Yu Wang², Monique Rivera³, Mamoudou Setamou⁴,
and Lukasz L. Stelinski^{1*}

¹ Department of Entomology and Nematology, Citrus Research and Education Center, University of Florida, Lake Alfred, Florida 33850, United States of America

² Department of Food Science and Human Nutrition, Citrus Research and Education Center, University of Florida, Lake Alfred, Florida 33850, United States of America

³ Department of Entomology, University of California Riverside, Riverside, California 92521, United States of America

⁴ Texas A&M University-Kingsville Citrus Center, 312 N International Blvd, Weslaco, Texas, 78599, United States of America

*Corresponding authors: Email: stelinski@ufl.edu and fbanezcarrasco@ufl.edu

Other authors: Email: Mamoudou.Setamou@tamuk.edu, yu.wang@ufl.edu, monique.rivera@ucr.edu, and joonhyuksuh@ufl.edu

Full contact corresponding authors:

Department of Entomology and Nematology, Citrus Research and Education Center, University of Florida, Lake Alfred, FL 33850, USA. Phone number +1 863 956 8852.

Abstract

Plant immunity against pathogens and pests is comprised of complex mechanisms orchestrated by signaling pathways regulated by plant hormones [Salicylic acid (SA) and Jasmonic acid (JA)]. Investigations of plant immune response to phytopathogens and phloem-feeders have revealed that SA plays a critical role in reprogramming of the activity and/or localization of transcriptional regulators via post-translational modifications. We explored the contributing effects of herbivory by a phytopathogen vector [Asian citrus psyllid, *Diaphorina citri*] and pathogen [*Candidatus Liberibacter asiaticus* (CLas)] infection on response of sweet orange [*Citrus sinensis* (L.) Osbeck] using manipulative treatments designed to mimic the types of infestations/infections that citrus growers experience when cultivating citrus in the face of Huanglongbing (HLB) disease. A one-time (7 d) inoculation access period with CLas-infected vectors caused SA-associated upregulation of *PR-1*, stimulating defense response after a long period of infection without herbivory (270 and 330 days). In contrast, while repeated (monthly) ‘pulses’ of 7 d psyllid feeding injury stimulated immunity in CLas-infected citrus by increasing [SA] in leaves initially (up to 120 d), long-term (270 and 330 days) repeated herbivory caused [SA] to decrease coincident with upregulation of genes associated with SA metabolism (*BMST* and *DMR6*). Similarly, transcriptional responses and metabolite (SA and its analytes) accumulation in citrus exposed to a continuously reproducing population of *D. citri* exhibited a transitory upregulation of genes associated with SA signaling at 120 days and a posterior downregulation after long-term psyllid (adults and nymphs) feeding (270 and 330 days). Herbivory played an important role in regulation of SA accumulation in mature leaves of *C. sinensis*, whether or not those

trees were coincidentally infected with CLAs. Our results indicate that prevention of feeding injury inflicted by *D. citri* from the tritrophic interaction may allow citrus plants to better cope with the consequences of CLAs infection, highlighting the importance of vector suppression as a component of managing this cosmopolitan disease.

Author Summary

We explored tritrophic interactions among an insect vector (*Diaphorina citri*) – phytopathogen (*Candidatus Liberibacter asiaticus*) – and cultivated fruit crop [sweet orange, *Citrus sinensis* (L) Osbeck]. Transcriptional and metabolic responses of plants were analyzed over an extended time-course of disease progression after various frequencies of herbivore feeding and durations of pathogen infection using manipulative treatments designed to mimic the types of infestations/infections that citrus growers experience when cultivating citrus in the presence of the devastating citrus disease, huanglongbing. We found that in the absence of coincident psyllid feeding damage, citrus trees could mount a defense response against the pathogen by activating the salicylic acid (SA) pathway. Repeated, monthly ‘pulses’ of herbivory led to pronounced stimulation of SA transcription that was coincident with diminished pathogen titers in plants. Although insect injury initially activated SA-dependent defense responses, continuous and/or long-term (≥ 270 d) herbivory shut down *PR-1*-dependent defense responses against the pathogen. Our results provide a mechanism explaining how vector suppression contributes to maintaining health of cultivated citrus in areas where huanglongbing is endemic. Our results also point to specific gene targets that may yield novel genotypes expressing tolerance against CLAs after appropriate manipulations.

Introduction

Huanglongbing (HLB), also known as citrus greening is a disease that limits production of commercially important *Citrus* spp. worldwide [1]. Three taxonomic species of pathogenic bacteria are associated with HLB: *Candidatus Liberibacter asiaticus*, *C. Liberibacter africanus*, and *C. Liberibacter americanus*. In Florida, only *C. Liberibacter asiaticus* (CLas) has been reported and is transmitted by the vector, Asian citrus psyllid, *Diaphorina citri* Kuwayama [1, 2]. Despite efforts to control the disease and/or vector, current strategies have had limited effect on curtailing disease spread resulting in lost commercial feasibility. Florida's citrus industry has been devastated losing over \$7 billion [3], putting the future of America's citrus production capabilities at risk. Nevertheless, significant research effort targeting this problem is beginning to yield promising results, including identification of two varieties that exhibit levels of tolerance to CLas infection - 'Bearss' lemon, *Citrus limon* Burm. f., and 'LB8-9' Sugar Belle® mandarin hybrid ("Clementine" mandarin × "Minneola" tangelo). Lower levels of phloem disruption and greater phloem regeneration have been documented in these two varieties following CLas infection as compared with HLB-susceptible sweet orange, *Citrus sinensis* (L.) Osbeck [4]. However, the molecular mechanisms involved in this tolerance are still not understood and the biological events involved in defense systems of *Citrus* varieties require further investigation.

Plant defense mechanisms are an adaptive response to combat injurious organisms. Two innate immunity strategies *in planta* have evolved to detect pathogens and their mechanisms have been extensively reviewed [5-7]. The first strategy (barrier) is known as microbe-associated molecular patterns (MAMPs) or pathogen-associated

molecular patterns (PAMPs) [8-10] wherein pattern-recognition receptors localized in the host-cell membrane interact with conserved molecules of different groups of microbes, including: lipids, carbohydrates, proteins, and other small molecules. This recognition activates PAMP-triggered immunity (PTI) leading to an array of defense responses [5]. The second strategy, effector-triggered immunity (ETI), involves the secretion of pathogen effectors that interplay with host proteins commonly called [resistance (R) genes]. Successful pathogens that suppress host PTI responses, for instance the hypersensitive response, secrete specific effectors that modulate programmed cell death activated during PTI [11]. Most R genes encode nucleotide-binding site, leucine-rich, repeat (NB-LRR) proteins [12, 13]; for example, BRASSINOSTEROID INSENSITIVE 1-ASSOCIATED KINASE 1 (BAK1), which is targeted by bacterial effectors to interrupt the PTI response [14-16]. Recognition of bacterial molecules by PAMP or MAMP pattern-recognition receptors induces an oxidative burst produced by NADPH oxidase encoded by *rbohD* [17]. Also, such recognition activates the salicylic acid (SA)-dependent signaling pathway [18].

Plants not only must defend themselves against potential pathogens, but also encounter numerous herbivores during their lifetime that modulate specific biochemical and molecular responses, which vary depending on the type of feeding and/or oviposition occurring on the plant [19]. Investigations of plant-insect interactions using aphids and whiteflies as models have described induction of the SA-dependent immune response in plants as reviewed by Walling (20) and references therein. Similar investigations focusing on psyllids have demonstrated congruent immune responses in plants [21-23].

Plants synthesize SA using isochorismate synthase and phenylalanine ammonia-lyase enzymes and their respective pathways; these processes are reviewed in Chen, Zheng (24) and D'Maris Amick Dempsey, Vlot (25) and references therein. Recently, a non-enzymatic reaction for SA accumulation was described in *Arabidopsis thaliana*, wherein avrPphB Susceptible 3 (PBS3) in the cytosol catalyzes the formation of Isochorismate-9-glutamate (ISC-9-Glu), which later spontaneously decays into SA [26]. Afterward, SA is enzymatically modified by glycosylation, methylation, amino acid conjugation, and hydroxylation [25, 27]. The function(s) of SA metabolites have not been completely elucidated; but, putative roles were ascribed in defense regulation [28].

During SA-dependent signaling, proteins with major immune defense roles have been described; among them, the transcription cofactor, Nonexpresser of Pathogenesis Related genes 1 (NPR1), is a critical protein in plant defense during SA-dependent signaling. After nuclear translocation, NPR1 cofactor interacts with TGA transcription factors [29], and is proposed to function as a co-activator of gene expression for systemic acquired resistance (SAR) [30, 31]. Within the SA signaling pathway, two NPR1 paralogues, NPR3 and NPR4, were initially shown to interact with NPR1 and function as E3 ligases to degrade NPR1 in a [SA]-regulated manner to activate defense-gene expression [32, 33]. However, a recent study showed that NPR3/NPR4 interacts with TGAs to inhibit defense-related gene expression when [SA] is low. During pathogen infection, SA increases and binds to NPR3/NPR4, which releases the transcriptional repression of defense genes, allowing SA to bind NPR1, which in turn, activates the transcription of defense genes. We examined the mechanism(s) of immune response in *C. sinensis* via accumulation of SA and its metabolites after 7-, 14-,

and 150-days of feeding by uninfected *D. citri*. Depending on the duration of herbivory, the vector differentially regulates transcription and accumulation of SA and its metabolites in mature leaves [23]. An increasing body of evidence suggests that immune responses against phloem-feeders and pathogens are tightly regulated by SA accumulation. We hypothesized that a threshold [SA] exists in *C. sinensis* that modulates the transcriptional regulation of genes involve in immune defenses and varies in accumulation in response to an interaction between stressors (pathogen and/or vector).

The overall goal of this study was to investigate modulation of SA-dependent plant defense responses in *C. sinensis* L. Osbeck (sweet orange). Three distinct frequencies of *D. citri* herbivory and CLas-inoculation access periods were imposed on *C. sinensis* as manipulated stressors. Our results describe the molecular and SA-dependent metabolic events that occur during HLB disease progression and after various intervals of insect feeding and following various frequencies of CLas inoculation. Specifically, we analyzed expression of a selected group of genes involved in: plant immune response, SA modification, and downstream SA signaling (perception). Concurrently, LC/MS analyses were conducted to quantify accumulation of SA and its metabolites. Based on our results, we propose working models to describe immune response in *Citrus* species following various intervals of *D. citri* infestation and frequencies of CLas inoculation.

Results

CLas detection in mature leaves of *C. sinensis*

The pattern of CLas-DNA titer in trees that were exposed to CLas-infected *D. citri* for one-time, 7-d inoculation access period (IAP) was similar to that found in trees that were exposed to a reproducing population of CLas-infected *D. citri* (presence of eggs, nymphs and adults) (Table 1). However, trees exposed to monthly pulses of 7 d IAPs using CLas-infected *D. citri* exhibited significantly lower CLas titer than the other two treatments for the duration of the experiment (12 months), with detectable levels of CLas DNA (0.010, 0.011 and 0.080 pg) at 240-, 330-, and 360-days, respectively. Based on these results, we chose time points when bacteria were detected in leaves (120-, 270-, and 360-days after first CLas-IAP) to analyze gene expression involved in SA-dependent defense response, as well as, accumulation of SA and SA-metabolites.

Table 1. Quantification of CLas genomic DNA in mature leaves of *C. sinensis* after initiation of CLas-IAP. The time-points chosen for gene expression and metabolite analyses were highlighted in bold, italicized, and underlined. Data represent mean $\pm$ standard deviation (SD) of six biological replicates per treatment. Different letters indicate statistical differences at $P < 0.05$. Abbreviation 'N.D.' denote No Detection of CLas DNA.

pg of CLas genomic DNA per 10 g of <i>C. sinensis</i> DNA			
Days after first CLas-IAP	One-time inoculation	Pulsed-inoculations	Continuous-inoculations
30	N.D.	N.D.	N.D.
60	N.D.	N.D.	N.D.
90	N.D.	N.D.	N.D.

<u>120</u>	0.715 ± 0.172 ^a	N.D.	0.308 ± 0.130 ^a
150	N.D.	N.D.	N.D.
180	N.D.	N.D.	N.D.
210	N.D.	N.D.	N.D.
240	N.D.	0.010 ± 0.009 ^b	N.D.
<u>270</u>	0.680 ± 0.101 ^a	N.D.	0.721 ± 0.270 ^a
300	N.D.	N.D.	0.041 ± 0.021 ^a
330	0.662 ± 0.271 ^a	0.011 ± 0.010 ^b	0.818 ± 0.081 ^a
<u>360</u>	2.325 ± 1.193 ^d	0.080 ± 0.005 ^c	2.047 ± 0.602 ^d

Gene expression and SA metabolite accumulation analyses

i) First scenario ‘One-time inoculation’

Transcriptional regulation of genes involved in SA signaling, and SA accumulation in mature leaves of *C. sinensis*.

Following the 7 days IAP treatment, the transcriptional regulation of *NPR1*, *NPR3*, *NPR4*, and *PR-1* and its association with endogenous SA levels were analyzed in leaves of CLas-infected and sham control plants (Fig 1). Quantitative RT-PCR (qRT-PCR) analysis indicated that the relative expression of *NPR1* was only significantly upregulated ($P = 0.035$) in CLas-infected mature leaves during the first instance of CLas detection (1st D) compared to the sham control (Fig 1A). The relative expression of *NPR3* was significantly upregulated (1st D, $P = 0.049$; 2nd D, $P = 0.045$; and 4th D, $P = 0.007$) during each of the three instances when CLas was detected in infected leaves as compared to non-infected samples (Fig 1B). However, the relative expression of *NPR4* was significantly upregulated ($P = 0.042$) in CLas-infected leaves only during the first instance of detection (1st D), and its temporal expression pattern was downregulated ($P < 0.05$) during the second instance of CLas detection (2nd D) compared to other time-

points (Fig 1C). The NPR-regulated gene, *PR-1*, was significantly upregulated during the second instance of CLas detection (2nd D) ($P = 0.004$) in CLas-infected leaves compared to sham control leaves (Fig 1D), and its temporal expression was significantly upregulated ($P < 0.05$) during the second (2nd D) and fourth (4th D) instances of CLas detection compared to the initial time when CLas was detected in this treatment.

The phytohormone SA binds to NPR proteins, modulating the transcription of the *PR-1* defense gene; significantly higher levels of SA were accumulated in CLas-infected leaves than in control leaves during the second (2nd D, $P = 0.006$) and fourth (4th D, $P = 0.049$) instances of detection compared to the initial time when CLas was detected in this treatment (Fig 1E).

Fig 1. SA signaling and accumulation after 'One-time inoculation'. CLas-infection induces changes in expression patterns of *NPR1*, *NPR3*, *NPR4*, and *PR-1*, as well as, SA accumulation in mature leaves of *C. sinensis*. Expression patterns of genes involved in SA signaling (A-D), SA accumulation in mature leaves (E). Expression levels were normalized using two references genes, *β-Actin* and *Elongation factor 1α*. Data represent mean ± standard deviation (SD) of six biological replicates per treatment. The gray dotted line denotes the level of SA accumulation at time zero in uninfected citrus

plants. White bars represent plants exposed to Non-infected (NI) *D. citri*, while black bars denote plants exposed to CLas-infected (I) *D. citri*.

Transcriptional regulation of genes involved in SA modifications and SA metabolite accumulation in mature leaves of *C. sinensis*.

qRT-PCR analysis indicated that *BSMT*, required for the methylation of SA into MeSA, was significantly upregulated in CLas-infected mature leaves during the second (2nd D, $P = 0.039$) and fourth (4th D, $P = 0.049$) instances of CLas detection compared to the sham control (Fig 2A). However, *MES1*, requiring esterification of MeSA into SA (active defense hormone), did not exhibit changes in gene expression between CLas-infected and CLas-free leaves (Fig 2B). *DMR6*, involved in hydroxylation of SA into 2,3- and 2,5-DHBA, was significantly upregulated in CLas-infected leaves during the second (2nd D, $P = 0.006$) and fourth (4th D, $P = 0.008$) instances of CLas detection compared to CLas-free leaves (Fig 2C).

MeSA was highly accumulated in CLas-infected leaves at the second (2nd D, $P = 0.015$) and fourth (4th D, $P = 0.032$) instances of CLas detection. The hydroxylated molecules of SA, 2,3- and 2,5-DHBA, exhibited a different temporal accumulation; the level of 2,3-DHBA was only significantly ($P = 0.02$) higher in CLas-infected than control leaves during the first (1st D) instance of CLas detection (Fig 2E). However, 2,5-DHBA was significantly accumulated in CLas-infected mature leaves during the second (2nd D, $P = 0.049$) and fourth (4th D, $P = 0.047$) instances of CLas detection compared to uninfected samples (Fig 2F).

Fig 2. SA modifications and metabolite accumulation after 'One-time inoculation'.

CLas-infection induces changes in expression pattern of *BSMT*, *MES1*, and *DMR6*, as well as, SA metabolite accumulation in mature leaves of *C. sinensis*. Expression patterns of genes involved in SA signaling (A-C), SA metabolite accumulation in mature leaves (D-F). Expression levels were normalized using two references genes, β -Actin and *Elongation factor 1 α* . Data represent mean $\pm$ standard deviation (SD) of six

biological replicates per treatment. White bars represent plants exposed to Non-infected (NI) *D. citri*, while black bars denote plants exposed to CLas-infected (I) *D. citri*.

Second scenario: ‘pulsed inoculations’

qRT-PCR analysis of leaves from *C. sinensis* trees exposed to repeated (monthly) pulses of 7 days IAP revealed that relative expression of *NPR1* was significantly downregulated in CLas-infected leaves during the second (2nd D, $P = 0.043$) and fourth (4th D, $P = 0.05$) instances of CLas detection compared with their respective controls. Also, there was a significant reduction in the expression of *NPR1* over time in both uninfected and infected mature leaves ($P < 0.05$, Fig 3A). Expression of *NPR3* was significantly downregulated in CLas-infected leaves at the fourth (4th D, $P = 0.045$) instance of CLas detection compared to the respective control (Fig 3B). However, expression of *NPR4* was upregulated in uninfected leaves during the second (2nd D, $P = 0.032$) instance of CLas detection relative to CLas-infected leaves (Fig 3C). Overall, expression of all *NPR* genes tended to decrease over time in mature leaves as a response to pulses of 7-day IAPs by CLas-infected psyllids. Expression of *PR-1* was only upregulated in CLas-infected mature leaves during the second (2nd D, $P = 0.003$) instance of CLas detection compared to its control.

Measurements of SA dynamics over time in trees that received pulses of 7-day IAPs revealed that [SA] was only significantly accumulated in CLas-infected mature leaves during the second (2nd D, $P = 0.049$) instance of CLas detection compared to uninfected leaves. When temporal accumulation of SA was analyzed, we found that [SA] increased during the first (1st D) instance of CLas detection in both uninfected and

infected leaves. Thereafter, there was a significant decline of [SA] in leaves observed during the second (2nd D, $P < 0.05$) and fourth (4th D, $P < 0.05$) instances of CLas detection (Fig 3E).

Fig 3. SA signaling and accumulation after 'Pulsed-inoculations'. CLas-infection induces changes in expression pattern of *NPR1*, *NPR3*, *NPR4*, and *PR-1*, as well as, SA accumulation in mature leaves of *C. sinensis*. Expression patterns of genes involved in SA signaling (A-D), SA accumulation in mature leaves (E). Expression levels were

normalized using two references genes, *β-Actin* and *Elongation factor 1α*. Data represent mean ± standard deviation (SD) of six biological replicates per treatment. The gray dotted line denotes the level of SA accumulation at time zero in uninfected citrus plants. White bars represent plants exposed to Non-infected (NI) *D. citri*, while black bars denote plants exposed to CLas-infected (I) *D. citri*.

Transcriptional regulation of genes involved in SA modifications and SA metabolite accumulation in mature leaves of *C. sinensis*.

The expression of *BSMT* was downregulated ($P = 0.049$) during the first (1st D time) instance of CLas detection in CLas-infected leaves as compared to that observed in its control (Fig 4A). *BSMT* did exhibit significant changes in expression among sampling points over time (Fig 4A). Expression of *MES1* did not differ between CLas-infected and uninfected leaves (Fig 4B) at any of the sampling points; however, its expression decreased over time and was significantly ($P < 0.05$) lower during the fourth (4th D) instance of CLas detection in CLas-infected than uninfected leaves. Conversely, *DMR6* was significantly downregulated ($P = 0.045$) in CLas-infected leaves during fourth (4th D) instance of CLas detection compared to that observed in uninfected leaves (Fig 4C).

In addition to transcriptional analysis, we examined the temporal accumulation of SA metabolites. [MeSA] did not differ significantly ($P > 0.05$) between CLas-infected and uninfected leaves during the specific time points when CLas was detected. However, there were significant ($P < 0.05$) changes in [MeSA] over time with a greater accumulation during the first (1st D) instance of CLas detection compared to the other two time points when the pathogen was detected (Fig 4D). The [2,3-DHBA] was

308 significantly lower in CLAs-infected than control leaves during the first (1st D, $P = 0.045$)
 309 and second (2nd D, $P = 0.041$) instances of CLAs detection. The [2,5-DHBA] was
 310 significantly higher in CLAs-infected than uninfected leaves during the second (2nd D, P
 311 = 0.0001) instance of CLAs detection (Fig 4F).
 312

313
 314 **Fig 4. SA modifications and metabolite accumulation after 'Pulsed-inoculations'.**
 315 CLAs-infection induces changes in expression pattern of *BSMT*, *MES1*, and *DMR6*, as

well as, SA metabolite accumulation in mature leaves of *C. sinensis*. Expression patterns of genes involved in SA signaling (A-C), SA metabolite accumulation in mature leaves (D-F). Expression levels were normalized using two references genes, *β-Actin* and *Elongation factor 1α*. Data represent mean ± standard deviation (SD) of six biological replicates per treatment. White bars represent plants exposed to Non-infected (NI) *D. citri*, while black bars denote plants exposed to CLas-infected (I) *D. citri*.

Third scenario: ‘continuous inoculations’

The transcriptional regulation of *C. sinensis* exposed to a continuously reproducing population of *D. citri* revealed that *NPR1* expression did not differ between CLas-infected and uninfected leaves at any of the time points when CLas was detected (Fig 5A). However, expression of *NPR1* was significantly ($P < 0.05$) lower during the second (2nd D) and fourth (4th D) instance of CLas infection as compared its expression when CLas was detected for the first time (Fig 5A). The expression of *NPR3* was upregulated in CLas-infected leaves during the first (1st D, $P = 0.046$) instance of CLas detection compared with that in its respective uninfected control (Fig 5B), and similarly to *NPR1*, its expression was downregulated during the second (2nd D) and fourth (4th D) instance of CLas detection in CLas-infected as compared with uninfected leaves (Fig 5B). In the case *NPR4*, there were no statistical differences between infected and uninfected plants exposed to reproducing populations of *D. citri* (Fig 5C). Expression of *NPR4* was significantly ($P < 0.05$) lower during the fourth (4th D) instance of CLas detection as compared its expression when CLas was detected for the first time (Fig 5C). Expression

of *PR-1* was significantly upregulated ($P < 0.001$) in uninfected than CLas-infected leaves during the second (2nd D) instance of CLas detection (Fig 5D).

The [SA] was higher in CLas-infected plants exposed to continuous re-inoculation and herbivory during the second (2nd D time) instance of CLas infection compared to the complementary control plants that were exposed to uninfected insects. However, the [SA] was significantly ($P > 0.05$) lower during the fourth (4th D) instance of CLas detection compared with [SA] recorded in both infected and uninfected leaves when CLas was detected for the first time (Fig 5E).

Fig 5. Signaling of SA and accumulation after 'Continuous-inoculations'. CLas-infection induces changes in expression pattern of *NPR1*, *NPR3*, *NPR4*, and *PR-1*, as well as, SA accumulation in mature leaves of *C. sinensis*. Expression patterns of genes involved in SA signaling (A-D), SA accumulation in mature leaves (E). Expression levels were normalized using two references genes, *β -Actin* and *Elongation factor 1 α* . Data represent mean $\pm$ standard deviation (SD) of six biological replicates per treatment.

White bars represent plants exposed to Non-infected (NI) *D. citri*, while black bars denote plants exposed to CLas-infected (I) *D. citri*.

Expression of *BMST* was significantly higher in CLas-infected than uninfected leaves during the second (2nd D) instance of CLas infection among plants that were continuously exposed to reproducing *D. citri* ($P = 0.002$, Fig 6A). The expression pattern of *MES1* did not differ between CLas-infected and uninfected samples; however, its expression was significantly upregulated ($P < 0.05$) during the second (2nd D) instance of CLas detection in both treatments as compared to that observed during the other sampling points (Fig 6B). The expression of the transcript involved in SA hydroxylation, *DMR6*, fluctuated over time with significantly ($P = 0.007$) higher expression during the second (2nd D) instance of CLas detection in CLas-infected than uninfected leaves and lower overall expression during the fourth (4th D) instance of CLas detection in both treatments ($P < 0.05$, Fig 6C).

The [MeSA] was significantly higher in CLas infected than uninfected leaves when both treatments were exposed to continuous herbivory only during the second (2nd D, $P < 0.05$) instance of CLas detection (Fig 6D). Of the hydroxylated [SA] metabolites, the [2,3-DHBA] was significantly higher ($P = 0.024$) in leaves of plants exposed to CLas-infected insects than in plants exposed to uninfected insects during the fourth (4th D) instance of CLas detection (Fig 6E). However, [2,5-DHBA] was only significantly higher in CLas-infected than uninfected leaves during the fourth (4th D) instance of CLas detection ($P = 0.001$), and there was a trend of progressively increasing 2,5-DHBA in leaves exposed to both infected and uninfected insects over time (Fig 6F).

Fig 6. SA modifications and metabolite accumulation after 'Continuous-inoculations'. CLas-infection induces changes in expression pattern of *BSMT*, *MES1*, and *DMR6*, as well as, SA metabolite accumulation in mature leaves of *C. sinensis*. Expression patterns of genes involved in SA signaling (A-C), SA metabolite accumulation in mature leaves (D-F). Expression levels were normalized using two references genes, β -Actin and *Elongation factor 1 α* . Data represent mean $\pm$ standard deviation (SD) of six biological replicates per treatment. Statistical analysis was

performed with repeated measures ANOVA and Tukey post-hoc tests. Different letters indicate significant differences between the samples ($P < 0.05$). White bars represent plants exposed to Non-infected (NI) *D. citri*, while black bars denote plants exposed to CLas-infected (I) *D. citri*.

Comparative analyses of SA and its analytes in response to the interaction of insect herbivory and pathogen infection

When the accumulated amount of SA and its metabolites were compared between all three scenarios investigated, there was a statistically significant increase of [SA] in CLas-infected leaves of plants exposed to infected *D. citri* as compared with the uninfected sham treatment in the OI scenario (Fig 7), suggesting a CLas-induced SA response. However, there was greater overall accumulation of SA in mature leaves from trees under the PI and CI scenarios than that accumulating in the OI scenario. Furthermore, when comparing between PI and CI scenarios, a difference in accumulation of SA between the CLas infection treatment and sham control was not apparent (Fig 7A).

Fig 7. Cumulative SA and metabolite concentrations among different scenarios of a vector-host-pathogen interaction in citrus. Data represent mean $\pm$ standard deviation (SD) of six biological replicates per treatment. Statistical analysis was performed with ANOVA with Tukey post-hoc tests. Different letters indicate significant differences between the samples ($P < 0.05$). White bars represent citrus plants exposed to uninfected psyllids, and black bars denote plants challenged with CLAs-infected psyllids, (OI = One-time inoculation, PI = Pulsed inoculations and CI = continuous inoculations).

Discussion

Plants perceive microbial and insect molecules as danger signals and mount defense against invasions. Studies investigating plant interactions with phytopathogens have established that plants have a layered innate immune system which responds to different microbial elicitors [5], and early signaling events are similar to those induced by insects [34-37]. The current investigation attempted to both disentangle the contributing effects of insect herbivory and pathogen infection on host plant response, as well as, gain insights into their interactive effects on disease progression. We inflicted insect and pathogen activity on citrus hosts with manipulative treatments that were designed to mimic the types of infestations/infections that citrus growers experience when cultivating citrus in the face of Huanglongbing (HLB). *C. sinensis* plants were challenged with three putative scenarios of vector-host-pathogen interactions [one time (OI), pulsed inoculation PI, and constant inoculation (CI) to explore transcriptional and metabolic modulation of SA-dependent defense responses in the host after varying frequencies of herbivore infestation and/or CLas inoculation. Moreover, the investigation was longitudinal, which allowed sampling of gene expression and metabolite accumulation over the course of disease progression.

Progression of pathogen in the host after trees were exposed to various frequencies of vector IAP (with or without pathogen present) was measured by monitoring CLas DNA. Simultaneously, we measured defense responses in *C. sinensis*, which may combat pathogenesis induced by CLas. Surprisingly, our results indicate that *C. sinensis* trees challenged by CLas-infected insects for only a 7d IAP exhibited similar temporal dynamics of CLas titer to trees that were continuously challenged with infected

D. citri for over 1 yr. In contrast, trees exposed to many (12-14), repeated 7d IAP challenges (pulsed-inoculations) by infected *D. citri* exhibited the lowest CLas titer, suggesting putative short-term plant defense responses to CLas (HLB progression, infection). We hypothesized that SA-dependent defense responses were active in these trees to reduce or ‘control’ CLas colonization.

Our results suggest that herbivory played an important role in inducing synthesis and accumulation of SA in mature leaves of *C. sinensis*, whether or not those trees are coincidentally infected with CLas. Accumulation of SA and the SA-signaling pathway play a critical role in plant defenses against pathogens [20] and in response to phloem-feeding herbivores [21-23]. SA regulates PTI and ETI defense mechanisms which leads to activation of systemic acquired resistance (SAR). We postulate that it might be eventually possible to manipulate natural defense and immune responses of *Citrus* to CLas and/or *D. citri* with sufficient understanding of the genes involved in SA-dependent immune response (*NPR1*, *NPR3*, *NPR4*, and *PR-1*) and genes that modulate downstream metabolic modifications (*BMST*, *MES1*, and *DMR6*).

Previously, a gene involved in the hydroxylation of SA, *SA hydroxylase*, was identified and characterized in CLas; infected plants exhibited significantly lower SA accumulation in leaves than uninfected plants 8 months after graft inoculation [38]. However, a similar reduction in SA was not observed in other investigations of the CLas-*C. sinensis* interaction. For example, Lu, Zhang (39) showed that symptomatic and PCR-positive leaves collected 3-4 months after graft-inoculation exhibited higher SA accumulation than uninfected trees. Similar results were detected after 6.4 months in young and mature leaves from trees previously graft-inoculated with CLas [40]. In the

first scenario we investigated, (OI) - trees without insects after initial IAP, significantly more SA accumulated in leaves from infected than uninfected trees at 9 and 12 months (Fig 1E). This outcome suggests that CLas did not evade SA-mediated immune responses via activity of its encoded SA hydroxylase within the context of this scenario where pathogen occurred *in planta* in the absence of concurrent insect herbivory/re-inoculation.

In addition to upstream regulation of SA, this hormone is also modulated by downstream metabolic modifications, including glycosylation, methylation, amino acid conjugation, and hydroxylation [25, 27]. In this investigation, production of the methylated (MeSA) and hydroxylated SA-metabolites (2,3- and 2,5-DHBAs) was measured longitudinally (Figs 2, 4 and 6) and their cumulative amounts were determined for the duration of the experiment (Fig 7 B-D). MeSA serves multiple functions, including triggering of SAR in response to microbial pathogens as shown with *Arabidopsis*, tobacco, and potato [41-44]. MeSA also functions as an indirect defense cue by attracting natural enemies in response to insect feeding [45-47]; e.g. MeSA attracts *D. citri* to citrus trees [48]. We measured substantial accumulation of MeSA in CLas-infected leaves (OI and PI). When CLas-infected vector IAP occurred in repeated pulses (the PI scenario), the titer of CLas in infected plants was the lowest of all three infection scenarios investigated (Table 1). In trees subjected to this IAP scenario, the concentration of MeSA varied over time and no difference in concentration was observed between trees injured by CLas-infected and uninfected insects (Fig 7B). In contrast, when plants were subjected to a one time IAP (OI) or a continuously breeding population of vectors (CI), the [MeSA] was substantially higher in leaves from trees that

were also challenged with the pathogen than in the sham controls that received only insect injury in the absence of CLas. These results are congruent with previous observations showing that CLas-infected field trees are attractive to *D. citri*, which is, in part, explained by MeSA serving an attractant cue or the vector (Mann et al. 2012).

Although the specific roles played by the hydroxylated SA molecules, 2,3- and 2,5-DHBA, in plant immune defense have not yet been clearly elucidated, studies suggest that exogenous applications of 2,3-DHBA in Arabidopsis induced weak *PR-1* expression [49]. Furthermore, 2,5-DHBA induced the synthesis of a different set of PR proteins in tomato [50]. We found no difference in 2,3-DHBA accumulation between trees that were subjected to sham (uninfected) IAPs versus CLas-infected IAPs, which resulted in eventual tree infection. However, more 2,3-DHBA accumulated in CLas-infected leaves that were subjected to a continuously breeding population of vectors (CI scenario) than in CLas-infected leaves from trees that were only injured by vectors during an initial one-time 7-d IAP (OI scenario, Fig 7C). In the case of 2,5-DHBA, it was highly accumulated in CLas-infected leaves (both OI and CI scenarios) compared to their respective sham controls, while the pulsed inoculation (PI scenario) caused intermediate accumulation with no difference observed between CLas-infected and uninfected (sham IAP) trees (Fig 7D). Based on our results, it appears that CLas infection induces the accumulation of 2,5-DHBA in *C. sinensis*; however, the effect of this analyte within the context of plant immune response is still unclear. The elucidation of this function was beyond the scope of our current study, but the role(s) of these hydroxylated SA molecules in *Citrus* should be investigated further.

Gene expression and its association with SA and related metabolites

We propose a working model of SA-modulated defense mechanisms in *C. sinensis* (S1 Fig) that examines the expression patterns of *NPR1*, *NPR3*, *NPR4*, *PR-1*, *BMST*, *MES1*, and *DMR6*, as well as, the accumulation of SA and its hydroxylated and methylated analytes based on the three scenarios of vector interactions with the host and pathogen (OI, PI, and CI).

One-time inoculation scenario. Using Arabidopsis and other plant species as models, it has been proposed that overexpression of *NPR1* induces expression of disease response genes, such as *PR-1* [51-53]. In our first scenario, CLas-infected samples exhibited a transient upregulation of *NPR1* in mature leaves only at 120 days after initial IAP and this coincided with the initial detection of CLas in the leaf phloem. However, upregulation of *NPR1* was not associated with a similar *PR-1* expression pattern as expected; *PR-1* was upregulated in CLas-infected leaves much later after initial IAP at 270- and 330- days, which was also much later than the initial detection of CLas titer in phloem of infected trees (day 120). Furthermore, this *PR-1* expression pattern was associated with high accumulation of SA in CLas-infected samples and reduced expression of *NPR4*. Recently, it was shown in Arabidopsis that the transcription factors NPR3/NPR4 interact with TGA2/TGA5/TGA6 to downregulate the expression of genes involved in plant defenses when plants are not infected and exhibit low SA concentration. Also, a gain-of-function mutation, *npr4-4D*, leads to a SA-insensitive mutant protein that represses SA-inducible defense genes [54]. Consequently, the expression pattern of *PR-1* is congruent with low expression of *NPR4* in association with SA accumulation in CLas-infected leaves. Therefore, we suggest that infection of *C. sinensis* with CLas activates SA-dependent defense responses even in the absence

of phloem feeding injury caused by the vector. However, characterizing how the pathogen induces this SA response in *C. sinensis* would likely require investigations employing gain/loss of function mutations, which will only be possible once this fastidious bacterium is reliably cultured [55-59].

Pulsed-inoculations scenario. For this inoculation scenario, trees were challenged by monthly ‘pulses’ of 7 d IAP inflicted by CLas-infected adult psyllids. To our surprise, these trees appeared to exhibit a much higher degree of ‘tolerance’ against CLas colonization, given that the lowest pathogen titers were measured in these trees when comparing across the three infection scenarios that we examined. In the pulsed inoculation scenario, SA-dependent immune responses and associated accumulation of SA and its metabolites were both substantial at 120 days after initial IAP, but then progressively decreased over time (Figs 4 and 5). We postulate that repeated exposure to insect injury caused sufficient accumulation of SA in *C. sinensis* to inhibit normal CLas colonization. Furthermore, we observed high expression of *PR-1* coincident with downregulation of *NPR1* and *NPR4* in trees exposed to CLas-infected insects by 270 days after initial IAP (Fig 3) even though no bacterial DNA from the pathogen was detected in leaf phloem at this point in the experiment (Table 1).

Continuous inoculations scenario. In this scenario, trees were challenged by the possibility of continuous CLas re-infection and constant injury inflicted by a reproducing population of *D. citri*. In general, the pattern of *NPR* transcripts differed from the OI scenario in that it decreased over time in both uninfected (sham inoculation receiving insect injury only) and CLas-infected trees; this downregulation of *NPR* response was also associated with lower SA accumulation than in the OI scenario. Specifically, trees

that were exposed to continuous feeding by *D. citri* initially accumulated SA (120 days after initial IAP), but SA production decreased significantly at later time-points (270- and 330- days after initial IAP). Injury to *C. sinensis* by *D. citri* feeding for brief intervals (≤ 14 days) and in the absence of CLas infection induces expression of *NPR1* and *PR-1*, but is not associated with changes in SA accumulation compared to control plants (without *D. citri*) [23]. In contrast, prolonged (≥ 150) feeding injury by uninfected *D. citri* stimulates accumulation of SA, yet no changes in expression of *NPR1* and *PR-1* were detected in these trees [23]. Collectively, these results indicate that psyllid feeding injury stimulates immune defenses in *C. sinensis* soon after feeding injury is initiated which can result in accumulation of SA, but prolonged feeding turns off SA synthesis and/or increases expression of genes involved in SA metabolism (*BMST* and *DMR6*), resulting in accumulation of SA metabolites (MeSA, 2,3-DHBA, and 2,5-DHBA) (Fig 7).

Our investigation describes gene expression associated with SA-dependent immune responses, as well as, resultant accumulation of SA and its metabolites in *C. sinensis* after various scenarios simulating infection of *Citrus* with the pathogen causing HLB. The investigation considered the interaction between vector and pathogen on host responses and varied duration of exposure to these two stressors. Based on our findings, we postulate that SA-dependent defense responses were activated in *Citrus* trees exposed to pulses of CLas inoculation at least until the initial instance of CLas detection in phloem. SA responses are of particular relevance to understanding HLB disease progression in sweet oranges, given that increased callose and phloem protein 2 deposition occurs in sieve elements of citrus trees expressing HLB symptoms [60, 61]. A recent investigation by Deng, Achor (4), showed that 'Bearss' lemon and 'LB8-9'

Sugar Belle® mandarin were more tolerant to CLas infection than most commercial citrus varieties, which was associated with lower levels of phloem disruption and greater phloem regeneration than in HLB-susceptible sweet orange. It is unclear whether or not SA was associated with this phenomenon; thus, it is imperative to understand the temporal accumulation of SA in citrus exposed to *D. citri* (vector) herbivory, as well as, CLas (pathogen) infection, and use this information to address fundamental questions related to symptom suppression and associated practical questions relating to developing tree therapy. Determining gene function involved in immune responses and SA modification in *Citrus* may yield novel genotypes that express tolerance against CLas and/or modify vector behavior to reduce inoculation.

The abnormal levels of gene expression and metabolite accumulation observed in trees challenged by prolonged feeding injury by *D. citri* appears to have a deleterious effect on the immune response of *C. sinensis*. From an immediately practical perspective, these findings support the practice of vector suppression as part of HLB management in citrus, even in areas where HLB is established endemically [23].

Methods

Controlled microcosm experiments:

Plant husbandry

All experiments were performed using of 2 year old *Citrus sinensis* L. Osbeck cv Valencia grafted onto US-812 rootstocks [62]. Plants were obtained from Southern Citrus Nurseries, Dundee, Florida. The process of eliminating possible insecticide residues from plants prior to initiation of experiments and in depth growing conditions

have been described previously [23]. In brief, plants were maintained at 23 ± 3 °C, 60RH, and a 16:8 h (Light: Dark) photoperiod. Plants were watered twice per week, and fertilized once per month with an alternating schedule of a 24-8-16 (Nitrogen–Phosphorus–Potassium) Miracle-Gro All Purpose Plant Food (Scotts Miracle-Gro Products, Marysville, OH) and a 10-10-10 (N–P–K) granular fertilizer (Growers Fertilizer Corp., Lake Alfred, FL).

Insect rearing conditions

Colonies of CLas-free and CLas-infected of *D. citri* were maintained on *C. sinensis* L. Osbeck cv Valencia trees in two separate greenhouses. Each greenhouse was maintained at 26 ± 2 °C, 60-65% RH, and a 16:8 h (Light: Dark) photoperiod with a maximum photosynthetic radiation of $215 \mu\text{mol s}^{-1} \text{m}^{-2}$. The CLas-infection rate was determined in each population monthly by testing a sub-sample of 40 adult insects per colony (infected and uninfected) using a TaqMan qPCR assay following the protocol described by Li, Hartung (63).

Inoculation frequency and herbivory duration treatments

Treatments were imposed on sweet orange, *C. sinensis*, seedling trees to simulate possible interactive scenarios of pathogen infection and insect herbivory that occur citrus groves with HLB. For each scenario below, 6 replicate trees were treated with CLas-infected or sham uninfected (control) psyllids. All trees (treatments and sham controls) were maintained individually in insect-proof cages (58.4 x 58.4 x 88.9 cm). Trees of similar phenology, characterized by presence of bud breaks and/or feather flush-like structures, were exposed to various frequencies of CLas-inoculation access periods (IAP) and duration of insect herbivory, as follows:

- i) First scenario: ‘one-time inoculation’ (OI):** Plants were exposed individually to groups of 20 CLas-infected (treatment) or CLas-free (control) *D. citri* adults (female: males; 1:1) for a 7d IAP. On day 7, all eggs laid on flush shoots and *D. citri* adults were gently removed and CLas-infection of adult psyllids was determined by TaqMan qPCR assay. This treatment simulated a scenario where a citrus grove is infested by a unique immigrant *D. citri* population that is eliminated by insecticide within one week under a zero-tolerance protocol.
- ii) Second scenario: ‘pulsed inoculations’ (PI):** Individual plants (N = 6 plants) were exposed to 20 CLas-infected (treatment) or CLas-free (sham control) *D. citri* adults for a 7d IAP. Psyllids were placed on branches within mesh sleeve cages. On the seventh day following insect release onto plants, all psyllids within cages were gently removed. This process was repeated monthly for the duration of the experiment (12 pulses of inoculations). In order to determine the CLas-infection rate of the adult insects throughout the course of this experiment, a subset of 40 psyllids were collected and examined by TaqMan qPCR assay during each month of the experiment; the rate of infection in psyllids varied between 35-72%. This treatment simulated a scenario where a citrus grove not harboring an endemic population of psyllids is periodically infested by immigrating psyllids, which are killed off by monthly applications of insecticides with short residual control.
- iii) Third scenario: ‘continuous inoculations’ (CI):** Individual plants were exposed to 20 CLas-infected (treatment) or CLas-free (sham control) *D. citri* by releasing psyllids into cages containing trees. In this treatment, insects were not removed and plants were constantly exposed to continuously reproducing *D. citri* populations. In

order to estimate the CLas-infection rate of the reproducing *D. citri* populations within cages, groups of insects (40 / tree) were randomly collected at three specific time-points between August (2018) and February (2019). This treatment was designed to simulate a scenario where a grove is continuously infested by an endemic population of *D. citri* that is never eliminated by management practices.

Plant tissue was sampled monthly from the same 12 mature leaves on each tree.

Samples were collected by harvesting discs from these leaves beginning at the leaf tip and progressing down the petiole on each sampled leaf as described in [64, 65]. Plant tissue samples were immediately flash frozen in liquid nitrogen, ground with a tissue lyser, and stored at -80°C for further analyses.

Genomic DNA extraction and CLas detection by TaqMan qPCR assays

To analyze the CLas infection rate in insects and plants, genomic DNA from individual *D. citri* adults and 20 mg of plant tissues was extracted using the DNeasy blood and tissue or DNeasy plant kits (Qiagen Inc, Valencia, CA), respectively. Both DNA extraction protocols were completed according to manufacturer's instructions. Quantity of genomic DNA was determined in a Nanodrop 2000 Spectrophotometer (Thermo fisher Scientific, Waltham, MA), and samples were stored at -20°C until TaqMan qPCR analysis were performed.

TaqMan assays were conducted using specific TaqMan probes previously designed by Li, Hartung (63) and targeting the 16S rDNA for CLas, *Wingless* for *D. citri*, and *Cytochrome oxidase I* for *C. sinensis*. TaqMan qPCR reactions and conditions were carried out as previously described [65]. TaqMan reactions were performed in duplicates and positive reactions were considered for either target sequence if the cycle

quantification (Cq) value was ≤ 36 . For CLas detection analysis, ‘undetermined values’ were automatically assigned with a Cq value equal to 45 cycles.

RNA extraction and cDNA synthesis

Total RNA extractions were carried out using 30 mg of ground plant tissue and the RNeasy Plant mini kit (Qiagen) according to the manufacturer’s instructions. To eliminate traces of genomic DNA, 1 μ g of total RNA per sample was treated with DNase I using the Turbo DNase kit (Ambion), following the manufacturer’s protocol. RNA quantity and purity were analyzed in a Nanodrop 2000 Spectrophotometer (Thermo fisher Scientific, Waltham, MA).

cDNA synthesis was executed using the Verso cDNA Synthesis kit (Thermo Fisher scientific, CA). Each reaction consisted of: 500 ng of total RNA, 5X cDNA synthesis buffer, anchored-Oligo (dT) primers, RT Enhancer, and Verso Enzyme Mix following the manufacturer’s instructions. After synthesis, samples were stored at -20°C until further analyses.

Selection of genes involved in SA signaling and metabolism

In order to examine the transcriptional regulation of seven genes involved in the SA-dependent defense responses on *C. sinensis*, we selected and evaluated four genes involved in the SA signaling; *Nonexpresser pathogenesis-related 1* (*NPR1*, accession number XM_006475416), *Nonexpresser pathogenesis-related 3* (*NPR3*, accession number XM_006468378), *Nonexpresser pathogenesis-related 4* (*NPR4*, accession number XM_025100491), and the *Pathogenesis-related 1* (*PR1*, accession number XM_006486757). Also, we examined three genes related to SA metabolism: *Methylesterase 1* (*MES1*, accession number NM_127926), *2-oxoglutarate (2OG)* and

Fe(II)-dependent oxygenase (Accession number NM_117118) annotated and described by [66] as a *salicylic acid 3-hydroxylase (DMR6)*, and *S-adenosyl-L-methionine-dependent methyltransferases (BSMT1)*, Accession number NM_111981). The expression patterns of these genes in response to feeding by uninfected *D. citri* were previously described [23]. All oligonucleotide primers for RT-qPCR analysis were designed using Primer3 web [67].

Table 2. Oligonucleotide primers used to examine gene expression by RT-qPCR.

Oligonucleotide primers		
Name	Sequence	Function
<i>Cs NPR1-like F</i>	5-TGATAAGACCTTGCCACAACAC-3	SA signaling
<i>Cs NPR1-like R</i>	5-ACCGCAGGATTCAGATCTATGT-3	
<i>Cs NPR3-like F</i>	5-GGAAGCCCTTATGAAAACAGTG-3	
<i>Cs NPR3-like R</i>	5-AAGGTCGTCCTCCATGAACTTA-3	
<i>Cs NPR4-like F</i>	5-TAAGGAGGCAAAGCAGCAAT-3	
<i>Cs NPR4-like R</i>	5-CTGCGATTAACCGCCACTGA-3	
<i>Cs PR1-like F</i>	5-ACTGCAATCTTGTGCATTCTG-3	
<i>Cs PR1-like R</i>	5-TTCACCCACAGTTTCACAGC-3	
<i>Cs BSMT-like F</i>	5-GGCTCTCAACAATATGGTCTCC-3	SA metabolism
<i>Cs BSMT-like R</i>	5-TTTATTTTCAGCTGGTGATGGTG-3	
<i>Cs DMR6-like F</i>	5-TCTTCCAGGTGAGGAACCAC-3	
<i>Cs DMR6-like R</i>	5-CTGGCGATTTGAAAAATGCT-3	
<i>Cs MES1-like F</i>	5-AGACACCAAACACCAACCATCT-3	
<i>Cs MES1-like R</i>	5-CTGAAAATTGAGTGTCACCA-3	
<i>Cs Actin F</i>	5-AGCCTACATTGCGCTTGACT-3	Reference genes
<i>Cs Actin R</i>	5-ATGACCTGCCCATCAGGTAG-3	

<i>Cs Elongation factor 1α F</i>	5-CCAAGGATGGTCAGACTCGT-3	
<i>Cs Elongation factor 1α R</i>	5-GCGTCCATCTTGTTACAGCA-3	

RT-qPCR reactions, gene expression, and metabolite analyses.

RT-qPCR reactions were performed using an Applied Biosystems 7500 Real-Time PCR System (Thermo Fisher Scientific). Each reaction consisted of: 10 ng of cDNA (template), 300 nM of each gene-specific primer (Table 2) and 1x of PowerUp™ SYBR® Green Master Mix; the final volume was adjusted with nuclease-free water to 20 μ L. The real-time PCR program, conditions, normalization, and mathematical estimations of relative expression at different time-points were conducted according to Ibanez, Suh (23).

Accumulated SA and SA analytes were extracted and analyzed following the protocol and conditions described in [23]. In brief, SA metabolites were extracted using 20 mg of ground leaves and each sample was mixed with 0.25 mL of ice-cold methanol/water solution (20/80, v/v), including three internal standards (0.01 μ g/mL salicylic acid-d₆, for salicylic acid; 0.4 μ g/mL 2,5-dihydroxybenzoic acid-d₃, and 2,3-dihydroxybenzoic acid; and 0.8 μ g/mL methyl salicylate-d₄ for methyl salicylate). Samples were processed by ultra-sonic assisted extraction for 30 min in an ice block-filled bathtub. After incubation, samples were centrifuged at 30,000 g at 4°C during 10 min, the supernatant was recovered and filtered with a 0.22 μ m membrane filter, and 10 μ L was injected into LC–MS/MS system. LC–MS/MS analyses were carried out with an Ultimate 3000 LC system coupled to a TSQ Quantiva triple quadrupole mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). The analytes (salicylic acid 2-O- β -D-glucoside, 2,3-dihydroxybenzoic acid, salicylic acid, and methyl salicylate)

were chromatographed on a Thermo Fisher scientific Acclaim C30 column (150 mm × 2.1 mm, 3.0 µm particle size) at a column temperature of 30°C using a gradient elution with 0.1% formic acid in water (eluent A) and 0.1% formic acid in acetonitrile (eluent B). The gradient was as follows: 0-10 min 20-95% B and 10-15 min 95% B. The column was re-equilibrated using the initial mobile phase before next run. The flow rate was set at 0.2 mL/min. The mass spectrometer was operated in both positive and negative electrospray ionization (ESI+ and ESI−) with selected reaction monitoring (SRM) mode. The analytes were assigned by comparing SRM transitions and retention times with authentic standards. Xcalibur software (Ver. 3.0) was employed for data processing and instrument control.

Statistical analyses

To compare the amount of CLas DNA in mature leaves between inoculation treatments, and to determine the differences in gene expression and accumulation of SA and its metabolites, repeated measures analysis of variance (ANOVA) with Tukey's test post hoc analyses were performed. All statistical analyses were run in RStudio environment [68].

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References

1. Bové JM. Huanglongbing: a destructive, newly-emerging, century-old disease of citrus. J Plant Pathol. 2006;7-37.
2. Manjunath K, Halbert S, Ramadugu C, Webb S, Lee R. Detection of ‘*Candidatus Liberibacter asiaticus*’ in *Diaphorina citri* and its importance in the management of citrus huanglongbing in Florida. Phytopathology. 2008;98(4):387-96.
3. Spreen TH, Baldwin J-P, Futch SH. An economic assessment of the impact of Huanglongbing on citrus tree plantings in Florida. HortScience. 2014;49(8):1052-5.
4. Deng H, Achor DS, Etxeberria E, Yu Q, Du D, Stanton D, et al. Phloem regeneration is a mechanism for Huanglongbing-tolerance of ‘Bearss’ lemon and ‘LB8-9’ Sugar Belle® mandarin. Front Plant Sci. 2019;10:277.
5. Jones JD, Dangl JL. The plant immune system. Nature. 2006;444(7117):323.
6. Boller T, Felix G. A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. Annu Rev Plant Biol. 2009;60:379-406.
7. Dodds PN, Rathjen JP. Plant immunity: towards an integrated view of plant–pathogen interactions. Nat Rev Genet. 2010;11(8):539.
8. Nicaise V, Roux M, Zipfel C. Recent advances in PAMP-triggered immunity against bacteria: pattern recognition receptors watch over and raise the alarm. Plant Physiol. 2009;150(4):1638-47.
9. Tsuda K, Katagiri F. Comparing signaling mechanisms engaged in pattern-triggered and effector-triggered immunity. Curr Opin Plant Biol. 2010;13(4):459-65.
10. Monaghan J, Zipfel C. Plant pattern recognition receptor complexes at the plasma membrane. Curr Opin Plant Biol. 2012;15(4):349-57.
11. Greenberg JT, Yao N. The role and regulation of programmed cell death in plant–pathogen interactions. Cell Microbiol. 2004;6(3):201-11.
12. McHale L, Tan X, Koehl P, Michelmore RW. Plant NBS-LRR proteins: adaptable guards. Genome Biol. 2006;7(4):212.
13. Dangl JL, Jones JD. Plant pathogens and integrated defence responses to infection. Nature. 2001;411(6839):826.

14. Chinchilla D, Zipfel C, Robatzek S, Kemmerling B, Nürnberger T, Jones JD, et al. A flagellin-induced complex of the receptor FLS2 and BAK1 initiates plant defence. *Nature*. 2007;448(7152):497.
15. Heese A, Hann DR, Gimenez-Ibanez S, Jones AM, He K, Li J, et al. The receptor-like kinase SERK3/BAK1 is a central regulator of innate immunity in plants. *PNAS*. 2007;104(29):12217-22.
16. Shan L, He P, Li J, Heese A, Peck SC, Nürnberger T, et al. Bacterial effectors target the common signaling partner BAK1 to disrupt multiple MAMP receptor-signaling complexes and impede plant immunity. *Cell host microbe*. 2008;4(1):17-27.
17. Zhang J, Shao F, Li Y, Cui H, Chen L, Li H, et al. A *Pseudomonas syringae* effector inactivates MAPKs to suppress PAMP-induced immunity in plants. *Cell host microbe*. 2007;1(3):175-85.
18. Glazebrook J. Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annu Rev Phytopathol*. 2005;43:205-27.
19. Mescher MC, De Moraes CM. Role of plant sensory perception in plant–animal interactions. *J Exp Bot*. 2014;66(2):425-33.
20. Walling LL. Avoiding effective defenses: strategies employed by phloem-feeding insects. *Plant Physiol*. 2008;146(3):859-66.
21. Casteel CL, Hansen AK, Walling LL, Paine TD. Manipulation of plant defense responses by the tomato psyllid (*Bactericera cockerelli*) and its associated endosymbiont *Candidatus Liberibacter psyllae*. *PloS one*. 2012;7(4):e35191.
22. Huot OB, Levy JG, Tamborindéguy C. Global gene regulation in tomato plant (*Solanum lycopersicum*) responding to vector (*Bactericera cockerelli*) feeding and pathogen (*Candidatus Liberibacter solanacearum*) infection. *Plant Mol Biol*. 2018;97(1-2):57-72.
23. Ibanez F, Suh JH, Wang Y, Stelinski LL. Long-term, sustained feeding by Asian citrus psyllid disrupts salicylic acid homeostasis in sweet orange. *BMC Plant Biol*. 2019;19(1):493.
24. Chen Z, Zheng Z, Huang J, Lai Z, Fan B. Biosynthesis of salicylic acid in plants. *Plant Signal Behav*. 2009;4(6):493-6.

- 801 25. D'Maris Amick Dempsey AC, Vlot MCW, Daniel FK. Salicylic acid biosynthesis
802 and metabolism. The Arabidopsis book/American Society of Plant Biologists. 2011;9.
- 803 26. Rekhter D, Lüdke D, Ding Y, Feussner K, Zienkiewicz K, Lipka V, et al. PBS3 is
804 the missing link in plant-specific isochorismate-derived salicylic acid biosynthesis.
805 bioRxiv. 2019:600692.
- 806 27. Maruri-López I, Aviles-Baltazar NY, Buchala A, Serrano M. Intra and extracellular
807 journey of the phytohormone salicylic acid. Front Plant Sci. 2019;10.
- 808 28. Huang X-x, Zhu G-q, Liu Q, Chen L, Li Y-j, Hou B-k. Modulation of plant salicylic
809 acid-associated immune responses via glycosylation of dihydroxybenzoic acids. Plant
810 Physiol. 2018;176(4):3103-19.
- 811 29. Zhou J-M, Trifa Y, Silva H, Pontier D, Lam E, Shah J, et al. NPR1 differentially
812 interacts with members of the TGA/OBF family of transcription factors that bind an
813 element of the PR-1 gene required for induction by salicylic acid. Mol Plant Microbe
814 Interact. 2000;13(2):191-202.
- 815 30. Wang D, Amornsiripanitch N, Dong X. A genomic approach to identify regulatory
816 nodes in the transcriptional network of systemic acquired resistance in plants. PLoS
817 Pathog. 2006;2(11):e123.
- 818 31. Delaney T, Friedrich L, Ryals J. Arabidopsis signal transduction mutant defective
819 in chemically and biologically induced disease resistance. PNAS. 1995;92(14):6602-6.
- 820 32. Fu ZQ, Yan S, Saleh A, Wang W, Ruble J, Oka N, et al. NPR3 and NPR4 are
821 receptors for the immune signal salicylic acid in plants. Nature. 2012;486(7402):228.
- 822 33. Yan S, Dong X. Perception of the plant immune signal salicylic acid. Curr Opin
823 Plant Biol. 2014;20:64-8.
- 824 34. Walling LL. The Myriad Plant Responses to Herbivores. J Plant Growth Regul.
825 2000;19(2):195-216.
- 826 35. Taylor JE, Hatcher PE, Paul ND. Crosstalk between plant responses to
827 pathogens and herbivores: a view from the outside in. J Exp Bot. 2004;55(395):159-68.
- 828 36. Garcia-Brugger A, Lamotte O, Vandelle E, Bourque S, Lecourieux D, Poinssot B,
829 et al. Early signaling events induced by elicitors of plant defenses. Mol Plant Microbe
830 Interact. 2006;19(7):711-24.

37. Maffei ME, Mithöfer A, Boland W. Before gene expression: early events in plant–insect interaction. *Trends Plant Sci.* 2007;12(7):310-6.
38. Li J, Pang Z, Trivedi P, Zhou X, Ying X, Jia H, et al. ‘*Candidatus Liberibacter asiaticus*’ Encodes a Functional Salicylic Acid (SA) Hydroxylase that Degrades SA to Suppress Plant Defenses. *Mol Plant Microbe Interact.* 2017(ja).
39. Lu H, Zhang C, Albrecht U, Shimizu R, Bowman K. Overexpression of a citrus NDR1 ortholog increases disease resistance in *Arabidopsis*. *Front Plant Sci* 2013;4:157.
40. Nehela Y, Hijaz F, Elzaawely AA, El-Zahaby HM, Killiny N. Citrus phytohormonal response to *Candidatus Liberibacter asiaticus* and its vector *Diaphorina citri*. *Physiol Mol Plant Pathol.* 2018;102:24-35.
41. Park S-W, Kaimoyo E, Kumar D, Mosher S, Klessig DF. Methyl salicylate is a critical mobile signal for plant systemic acquired resistance. *Science.* 2007;318(5847):113-6.
42. Vlot AC, Liu PP, Cameron RK, Park SW, Yang Y, Kumar D, et al. Identification of likely orthologs of tobacco *salicylic acid-binding protein 2* and their role in systemic acquired resistance in *Arabidopsis thaliana*. *Plant J.* 2008;56(3):445-56.
43. Vlot AC, Dempsey DMA, Klessig DF. Salicylic acid, a multifaceted hormone to combat disease. *Annu Rev Phytopathol.* 2009;47:177-206.
44. Manosalva PM, Park S-W, Forouhar F, Tong L, Fry WE, Klessig DF. Methyl esterase 1 (*StMES1*) is required for systemic acquired resistance in potato. *Mol Plant Microbe Interact.* 2010;23(9):1151-63.
45. Van Poecke RM, Posthumus MA, Dicke M. Herbivore-induced volatile production by *Arabidopsis thaliana* leads to attraction of the parasitoid *Cotesia rubecula*: chemical, behavioral, and gene-expression analysis. *J Chem Ecol.* 2001;27(10):1911-28.
46. Simpson M, Gurr GM, Simmons AT, Wratten SD, James DG, Leeson G, et al. Insect attraction to synthetic herbivore-induced plant volatile-treated field crops. *Agric For Entomol.* 2011;13(1):45-57.
47. Martini X, Pelz-Stelinski KS, Stelinski LL. Plant pathogen-induced volatiles attract parasitoids to increase parasitism of an insect vector. *Front Ecol Evol.* 2014;2:8.

48. Mann RS, Ali JG, Hermann SL, Tiwari S, Pelz-Stelinski KS, Alborn HT, et al. Induced release of a plant-defense volatile 'deceptively' attracts insect vectors to plants infected with a bacterial pathogen. *PLoS Pathog.* 2012;8(3):e1002610.
49. Bartsch M, Bednarek P, Vivancos PD, Schneider B, von Roepenack-Lahaye E, Foyer CH, et al. Accumulation of isochlorogenic acid-derived 2, 3-dihydroxybenzoic 3-O- β -D-xyloside in Arabidopsis resistance to pathogens and ageing of leaves. *J Biol Chem.* 2010;285(33):25654-65.
50. Bellés JM, Garro R, Fayos J, Navarro P, Primo J, Conejero V. Gentisic acid as a pathogen-inducible signal, additional to salicylic acid for activation of plant defenses in tomato. *Mol Plant Microbe Interact.* 1999;12(3):227-35.
51. Cao H, Bowling SA, Gordon AS, Dong X. Characterization of an Arabidopsis mutant that is nonresponsive to inducers of systemic acquired resistance. *The Plant Cell.* 1994;6(11):1583-92.
52. Chern M, Fitzgerald HA, Canlas PE, Navarre DA, Ronald PC. Overexpression of a rice *NPR1* homolog leads to constitutive activation of defense response and hypersensitivity to light. *Mol Plant Microbe Interact.* 2005;18(6):511-20.
53. Le Henanff G, Farine S, Kieffer-Mazet F, Miclot A-S, Heitz T, Mestre P, et al. *Vitis vinifera VvNPR1. 1* is the functional ortholog of *AtNPR1* and its overexpression in grapevine triggers constitutive activation of *PR* genes and enhanced resistance to powdery mildew. *Planta.* 2011;234(2):405-17.
54. Ding Y, Sun T, Ao K, Peng Y, Zhang Y, Li X, et al. Opposite roles of salicylic acid receptors NPR1 and NPR3/NPR4 in transcriptional regulation of plant immunity. *Cell.* 2018;173(6):1454-67. e15.
55. Davis MJ, Mondal SN, Chen H, Rogers ME, Bransky RH. Co-cultivation of '*Candidatus Liberibacter asiaticus*' with actinobacteria from citrus with huanglongbing. *Plant Dis.* 2008;92(11):1547-50.
56. Sechler A, Schuenzel E, Cooke P, Donnua S, Thaveechai N, Postnikova E, et al. Cultivation of '*Candidatus Liberibacter asiaticus*', '*Ca. L. africanus*', and '*Ca. L. americanus*' associated with huanglongbing. *Phytopathology.* 2009;99(5):480-6.
57. Tyler HL, Roesch LF, Gowda S, Dawson WO, Triplett EW. Confirmation of the sequence of '*Candidatus Liberibacter asiaticus*' and assessment of microbial diversity in

892 Huanglongbing-infected citrus phloem using a metagenomic approach. Mol Plant
893 Microbe Interact. 2009;22(12):1624-34.

894 58. Parker JK, Wisotsky SR, Johnson EG, Hijaz FM, Killiny N, Hilf ME, et al. Viability
895 of '*Candidatus Liberibacter asiaticus*' prolonged by addition of citrus juice to culture
896 medium. Phytopathology. 2014;104(1):15-26.

897 59. Ha PT, He R, Killiny N, Brown JK, Omsland A, Gang DR, et al. Host-free biofilm
898 culture of "*Candidatus Liberibacter asiaticus*," the bacterium associated with
899 Huanglongbing. Biofilm. 2019;1:100005.

900 60. Achor D, Etxeberria E, Wang N, Folimonova S, Chung K, Albrigo L. Citrus
901 affected with huanglongbing disease. Plant Pathol J. 2010;9:56-64.

902 61. Koh E-J, Zhou L, Williams DS, Park J, Ding N, Duan Y-P, et al. Callose
903 deposition in the phloem plasmodesmata and inhibition of phloem transport in citrus
904 leaves infected with "*Candidatus Liberibacter asiaticus*". Protoplasma. 2012;249(3):687-
905 97.

906 62. Bowman KD, Rouse RE. US-812 citrus rootstock. HortScience. 2006;41(3):832-
907 6.

908 63. Li W, Hartung JS, Levy L. Quantitative real-time PCR for detection and
909 identification of *Candidatus Liberibacter* species associated with citrus huanglongbing. J
910 Microbiol Methods. 2006;66(1):104-15.

911 64. Etxeberria E, Gonzalez P, Vincent C, Schumann A. An Improved Method to
912 Track Changes of *Candidatus Liberibacter asiaticus* Titer in HLB-affected Citrus Trees:
913 Extended Persistence of Detectable CLas DNA after Cell Death. Proceedings of the
914 Florida State Horticultural Society. 2017;130.

915 65. Ibanez F, Stelinski LL. Temporal Dynamics of *Candidatus Liberibacter asiaticus*
916 Titer in Mature Leaves from *Citrus sinensis* cv Valencia Are Associated with Vegetative
917 Growth. J Econ Entomol. 2019.

918 66. Zhang K, Halitschke R, Yin C, Liu C-J, Gan S-S. Salicylic acid 3-hydroxylase
919 regulates Arabidopsis leaf longevity by mediating salicylic acid catabolism. PNAS.
920 2013;110(36):14807-12.

921 67. Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, et al.
922 Primer3-new capabilities and interfaces. Nucleic Acids Res. 2012;40(15):e115-e.

68. RStudio. RStudio: integrated development for R. RStudio, Inc, Boston, MA URL
<http://www.rstudio.com>. 2015.

Supporting information

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950 **S1 Fig.** Proposed models of SA-dependent immune responses in *C. sinensis*.