Two Liberibacter Proteins Combine to Suppress Critical Innate Immune Defenses in Citrus

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We adopted a systems-based approach to determine the role of two Candidatus Liberibacter asiaticus (CLas) proteins, LasP235 and Effector 3, in Huanglongbing (HLB) pathogenesis. While a published work suggests the involvement of these CLas proteins HLB pathogenesis, the exact structure-based mechanism of their action has not been elucidated. We conducted the following experiments to determine the structure-based mechanisms of action. First, we immunoprecipitated the interacting citrus protein partners of LasP235 and Effector 3 from the healthy and CLas-infected Hamlin extracts and identified them by Liquid Chromatography with tandem mass spectrometry (LC–MS/MS). Second, we performed a split green fluorescent protein (GFP) assay in tobacco to validate that the interactions observed in vitro are also retained in planta. The notable in planta citrus targets of LasP235 and Effector 3 include citrus innate immune proteins. Third, in vitro and in planta studies were performed to show that LasP235 and Effector 3 interact with and inhibit the functions of multiple citrus proteins belonging to the innate immune pathways. These inhibitory interactions led to a high level of reactive oxygen species, blocking of bactericidal lipid transfer protein (LTP), and induction of premature programmed cell death (PCD), all of which are beneficial to CLas lifecycle and HLB pathogenesis. Finally, we performed molecular dynamics simulations to visualize the interactions of LasP235 and Effector 3, respectively, with LTP and Kunitz protease inhibitor. This led to the design of an LTP mimic, which sequestered and blocked LasP235 and rescued the bactericidal activity of LTP thereby proving that LasP235, indeed, participates in HLB pathogenesis.

Keywords: CLas protein, citrus immunity, HLB pathogenesis, ROS, PCD, bactericidal activity

INTRODUCTION

Huanglongbing (HLB) is the most devastating citrus disease (Da Graça et al., 2016; Merfa et al., 2019; Gupta and Stover, 2022). The Candidatus Liberibacter asiaticus (CLas) colonizes the phloem sieve elements on getting transmitted to the plants during the sap feeding by Asian citrus psyllid (ACP). The CLas infection in citrus plants leads molting of leaves and premature fruit drop. Three α-proteobacteria species
The gram-negative bacteria secrete effector proteins that play an essential role in disease pathogenesis by suppressing multiple proteins belonging to the innate immune system in plants and thereby providing a niche for bacterial colonization and spread in the host (Dodds and Rathjen, 2010). Typically, these effectors are directly injected into the host by the type III secretion system (Feng and Zhou, 2012). The CLas is devoid of the type III secretion system (Mudgett, 2005; Feng and Zhou, 2012) but may alternately use type II secretion system to release potential virulence factors or effectors (Sugio et al., 2011; Solé et al., 2015; Cianciotto and White, 2017). These proteins can be encoded by the CLas genome or the prophage. The prophages have been shown to exert influence in bacterial pathogenicity as have been seen in Staphylococcus aureus (Bae et al., 2006; Zhang et al., 2011). Two autotransporter proteins (LasA and LasAII) with leucine-rich repeats (LRRs) have been identified in Las psy62 prophage regions and have been shown to target the mitochondria in plants (Hao et al., 2013). Note that CLas codes for a smaller number of effectors because of the small (1 Mb) genome-size (Duan et al., 2009; Lin et al., 2013). The gram-negative bacteria with 5 Mb genomes have several 100 unique effectors (Dillon et al., 2019) as opposed to about 80 effectors identified, so far, from the CLas genome (Pitino et al., 2016, 2018; Prasad et al., 2016). However, the interactome studies revealed the ability of a single effector to bind multiple protein from the host plant (Block et al., 2008; Büttner, 2016). Thus, the bacteria have evolved to utilize a smaller number of effectors to establish a niche for pathogenesis in the host. Therefore, it was of importance whether to determine the two CLas proteins, LasP235 and Effector 3, may target multiple citrus proteins, suppress immunity, and contribute to HLB pathogenesis.

In this study, we focused first on identifying the critical steps associated with the breakdown of citrus innate immune defense in response by the CLas effectors. Typically, the plant innate immune defense involves multiple pathways including pathogen or microbe-associated molecular pattern (MAMP)-triggered immunity (MTI); pattern-triggered immunity (PTI), effector triggered immunity (ETI), and plant hormone, such as salicylic acid (SA), jasmonic acid (JA), and ethylene (ET), induced immunity (He et al., 2007; Wu L. et al., 2014; Brauer et al., 2018; Qi et al., 2018; Zhang et al., 2018; Alhoraibi et al., 2019). The PTI or MTI provides the first line of plant defense against pathogens or microbes through the recognition of PAMP or MAMP, such as bacterial lipopolysaccharide (LPS), elongation factor thermal unstable (EF–Tu), flagellin. The PAMP or MAMP recognition is mediated by the plasma membrane pattern recognition receptors (PRR) that include LRR, flagellin receptor (FLS2), and EF–Tu receptor (EFR). The plasma membrane PRR recognition induces intracellular mitogen-associated protein kinase (MAPK) signaling leading to the expression of pathogen-related (PR) or defense genes (Dangl and Jones, 2001; Sels et al., 2008; Ali et al., 2018). However, the pathogen effectors can block both intracellular and extracellular steps in the PTI pathway (Cui et al., 2009; Büttner, 2016). To counter the pathogen induced blocking of the PTI pathway, the plants have evolved the ETI pathway in which the intracellular nod-like receptors (NLR) recognize the pathogen effectors and augment the MAPK signaling and PR gene expression. The ETI pathway also induces hypersensitive response through the production of reactive oxygen species (ROS), which causes cell death at the site of the infection thereby limiting the pathogen spread. The PTI and ETI pathways also couple to intracellular plant hormone SA/JA/ET pathways, which also involve ROS production and induction of PR genes. It has been demonstrated that the effectors from plant pathogenic bacteria can inhibit one or more steps in these pathways (Medina et al., 2018; Mine et al., 2018; Han and Kahmann, 2019; Lee et al., 2019). Also, the bacterial effectors are known to subvert multiple steps leading to programmed cell death (PCD) in plant, which is a form of immune defense by PTI and/or ETI to control infection (Hoebertschts and Woltering, 2003; Abramovitch and Martin, 2005; Locato and De Gara, 2018). Therefore, it was of interest for us to determine which steps in the citrus innate immune defense are affected by the proteins encoded by the CLas genome and prophage.

First, we performed in vitro and in planta studies to identify the prominent citrus proteins targeted by LasP235 and Effector 3. Second, we performed functional assays to determine whether LasP235 and Effector 3 have inhibitory effects on their citrus protein targets. Third, we performed molecular dynamic simulations to analyze the details of interaction between LasP235 and Effector 3 and their selected citrus targets and predicted which pairwise interactions are critical for inhibition of the citrus target function. Finally, we validated our prediction of the inhibitory mechanism by site-specific mutations on the citrus protein(s) that affect the critical pairwise interactions. We discovered that each of the two effectors can directly target several citrus innate immune proteins. A clear understanding of the inhibitory mechanisms will provide guidelines for countering CLas effectors and developing anti-infectives to block HLB pathogenesis.

MATERIALS AND METHODS
Experimental Procedures
Plant Materials and Growth Conditions
Hamlin trees verified as being HLB-free and ACP-friendly were purchased and placed in the greenhouse. One branch cage placed in the upper part of each tree (three replicates) was filled with 75ACP from an infected population while other trees had cages with clean 75ACPs placed serving as control. The insects were allowed to feed on the trees for a week and then the insects were killed by spraying with topical insecticide. The ACPs were tested for CLas and the trees were subsequently returned to the greenhouse. The leaf samples (12 for each biological replicate and

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Cloning and Overexpression of Effectors and Targets in Escherichia coli

The genes from Liberibacter asiaticus (non-culturable bacteria) were identified (LasP235 and Effector 3), codon optimized and cloned in pUC57 by GenScript. The effectors were then amplified and cloned in pET28(a) vector between NdeI and BamHI sites and transformed in E. coli BL21 [BL21(DE3)pLysS]. The positive clones were inoculated overnight in LB with Kanamycin. The overnight culture (1%) was grown until the Optical density (OD) reached 0.6 and then induced with IPTG at 30°C for overnight.

The cells were harvested next day and resuspended in protein isolation buffer (20-mM Tris-Cl, pH 7.4, 150-mM NaCl, and 10% glycerol). The cell suspension was sonicated and centrifuged at 14,000 rpm, 4°C, 30 min. The supernatant was collected and the inclusion bodies were treated with 9M urea. Following the treatment with urea, the cell suspension was centrifuged and supernatant was collected and refolded. The refolded protein from the inclusion bodies and the soluble fractions were purified using TALON metal affinity resin (Joshi and Puri, 2005).

Isolation of Total Protein From Citrus

Fresh leaf tissue, from five Hamlin trees (Citrus sinensis L. Osbeck) was pulverized in liquid nitrogen using a pestle and mortar and the resulting fine powder stirred with 1.5 volumes of extraction buffer [50-mM HEPES pH 7.5, 5-mM EDTA, 5-mM EGTA, 10-mM dithiothreitol (DTT), 10% glycerol, 7.5% polyvinylpolypyrrolidone (PVPP), and a protease inhibitor cocktail, Complete™, Boehringer Mannheim]. The slurry was subsequently mixed on a reciprocating shaker (100 oscillations per min) for 10 min, at 4°C, followed by centrifugation 15,000 g for 30 min at 4°C. The supernatant was removed and immediately flash-frozen in liquid nitrogen and stored at −80°C until needed (Roy et al., 2011).

Pull Down Assay and LC–MS/MS Analysis to Identify Citrus Targets

The purified refolded effector proteins were incubated with total protein (15 µg) isolated from healthy and infected citrus leaf extract for 2 h at 4°C. The effector–protein complex was incubated with TALON metal affinity resin at 4°C overnight. The resin was washed with column buffer (50-mM Tris-Cl, pH 7.4, 150 mM, 10% glycerol) and eluted with imidazole (250 mM). The eluted protein complex was sent for LC–MS/MS analysis to identify the citrus targets (Zhang et al., 2017). The spectra were searched against the Uniprot database, and taxonomy was set to C. sinensis. The only peptides that were ranked 1 were selected and finally those targets were selected for further analysis that had a 95% confidence (Karpievitch et al., 2012).

Enzymatic Assays and Their Inhibitions by the CLas Effectors

Superoxide Dismutase Assay

The superoxide dismutase (SOD) assay was quantified based on its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) by superoxide radical and assayed following (SOD Kit; catalog No.: 7,500-100-K) with some modifications. The reaction mixture (3 ml) contained 13 mM methionine, 75-mM NBT, 2-mM riboflavin,100-mM EDTA, and 0.3-ml leaf extracts. The volume was made up to 3 ml using 50-mM phosphate buffer with the addition of riboflavin at the very end. Once the reaction mixture was made, they were mixed well and incubated below two 15-W fluorescent tubes with a photon flux density of around 40 mmol m−2 s−1 for 10 min. Once the reaction is completed, the tubes were covered with a black cloth and the absorbance was measured at 560 nm. The non-irradiated mixture served as control and the absorbance so measured is inversely proportional to the amount of enzyme added. The SOD activity is defined as the amount of enzyme that caused 50% inhibition of the enzymatic reaction in the absence of the enzyme (Basu et al., 2010). The presented data were an average of three biological replicates, and two of them were of technical replicates.

Aspartyl Protease Assay

The protease assay was performed using a fluorescence based (BODIPY) EnzChek protease assay kit. The analysis of aspartyl protease activity was done by incubating it with no other proteins in sodium citrate buffer (50 mM, pH 4.5). To perform the inhibitory effect of LasP235 on the protease activity the renatured aspartyl protease was preincubated with increasing concentrations of LasP235 at 4°C for 2 h in sodium citrate buffer. Following incubation, BODiPy-labeled casein substrate was added, and the reaction was monitored by measuring fluorescence in Tecan Infinite 200 PRO microplate reader at 485 ± 12.5 nm excitation/530 ± 15 nm emission filter. The assays were conducted in triplicates (Leippe et al., 2011; Coria et al., 2016).

Glycosyl Hydrolase Assay

The inhibitory effect of recombinant LasP235 on recombinant glycosyl hydrolase was assayed using the β-Glucosidase Activity Assay Kit (MAK129, Sigma). The enzymatic reactions were carried out in K-Phosphate buffer (100 mM, pH 6.5) with p-nitrophenyl-β-D-glucopyranoside (β-NPG) for 20 min at 37°C. The final absorbance of the hydrolyzed product was measured at 405 nm (Henriques et al., 2017).

Aldehyde Dehydrogenase Assay

This assay was performed using aldehyde dehydrogenase (ALDH) Activity Abcam Assay Kit with modifications. In short, the purified ALDH was incubated with increasing concentration of substrate (acetaldehyde) for 1 h. The absorbance was measured at 450 nm and expressed in terms of NADH standard as µM/ml (Ouyang et al., 2019). The presented data were an average of three biological replicates, and two of them were of technical replicates.

Trypsin Inhibition Assay

The trypsin inhibition assay was done in triplicate and the result was expressed as a means of three replicates. In short, the residual trypsin activity was measured by monitoring the change in absorbance at 247 nm in presence of increasing concentration of recombinant purified Kunitz Trypsin inhibitor (KTI) when incubated with p-toluenesulfonyl-L-Arg methyl ester (Sigma;
The presented data were an average of three biological replicates, and two of them were of technical replicates.

**In planta Split GFP Assay (Agro-Infiltration)**

*Agrobacterium tumefaciens* LBA4404 transformant cells carrying LasP<sub>235</sub>. Effector 3 and the targets from citrus plants (Aspartyl protease, glycosyl hydrolase, SOD, KTI protein, lectin etc.), respectively, are cloned in pR101 vector and cultured overnight in LB medium with 50 µg ml<sup>-1</sup> of rifampicin and 50 µg ml<sup>-1</sup> kanamycin, and resuspended in 10-mM MgCl<sub>2</sub>, 10-mM MES. The culture was diluted to an optical density of 0.5 (OD 600 nm). For each effector–target interaction, three leaves of *Nicotiana benthamiana* plants overexpressing GFP1-9 were infiltrated with the *A. tumefaciens* suspension containing the effector and the target plasmids, respectively. The agro-infiltrated leaves were analyzed for protein localization at 3 dpi under a microscope (Olympus BX51-P) equipped with a UV light source. The agroinfiltrated leaves were kept in a greenhouse for 24 h and the interaction was visualized using Illumatool lighting system (LT-9500; Lightools Research) with a 488-nm excitation filter (blue) and a colored glass 520-nm long pass filter. The photographs were taken by Photometric CoolSNAP HQ camera (Cabantous and Waldo, 2006; Liu et al., 2018).

**Estimation of Superoxide Anion**

The leaf disks from agro-infiltrated tobacco plants were incubated at 25°C on a shaker for 30 min in dark in 1 ml of K-phosphate buffer (20 mM, pH 6.0) containing 500-µM XTT. The increase in absorbance was measured at 470 nm in a spectrophotometer (Ramegowda et al., 2012). The presented data were an average of three biological replicates, and two of them were of technical replicates.

**Lipid Binding and MIC Assays for Lipid Transfer Protein**

The lipid binding activity of recombinant LTP-6X His protein overexpressed and purified from *E. coli* was mixed with 0.2-p-toluidinonaphthalene-6-sulphonate (TNS) at 25°C. The results were recorded at excitation 320 nm and the emission at 437 nm. The inhibitory action of LasP<sub>235</sub> on lipid transfer protein (LTP) was assessed using increasing concentration of LasP<sub>235</sub> and the results were measured. The purified GFP was used as a control (Melnikova et al., 2020). The minimum inhibitory concentration (MIC) of the LTP was performed using broth microdilution technique. The assay was carried out using 5 × 10<sup>5</sup> colony forming units (CFU ml<sup>-1</sup>) in MHB. The MIC was defined as the lowest concentration of the protein required to inhibit the visible growth of bacterial strains used (Ebbensgaard et al., 2015). The presented data were an average of three replicates, and two of them were of technical replicates.

**Estimation of Ion Leakage From Leaf Disks**

*Agrobacterium tumefaciens* LBA4404 transformant cells carrying Effector 3 and the targets from citrus plants KTI protein cloned in pR101 vector was cultured overnight in LB medium with 50 µg ml<sup>-1</sup> of rifampicin and 50 µg ml<sup>-1</sup> kanamycin and resuspended in 10-mM MgCl<sub>2</sub>, 10-mM MES. The culture was diluted to an optical density of 0.5 (OD 600 nm). For the assay, three leaves of *N. benthamiana* plants previously treated with paraquat (PQ; 100 µM) were infiltrated with the *A. tumefaciens* suspension containing the effector alone, Kunitz alone and the mixture of effector 3 and Kunitz, respectively (Pitino et al., 2016, 2018) and incubated for 48 h. The leaf disks were prepared by punching the leaf disks with a cork puncher. The punctured leaf disks were placed in water (50 ml) for 5 min to mitigate the error of measuring ion leakage due to injury inflicted on the leaves due to puncturing. The water was removed and the leaf discs were incubated with 5 ml. the conductivity was measured after 3 h using Mil80 bench meter and this value is referred to as A. The leaf disks with the bathing solution were then incubated at 95°C for 25 min and then cooled to room temperature to enable complete ion leakage. The conductivity was measured again, and this value is referred to as B. The ion leakage is subsequently expressed as (A/B) × 100. All experiments were carried out in three biological replicates with five leaf disks for each sample (Wu et al., 2017; Hatsugai et al., 2018).

**Pathogen Inoculation and LTP Treatment in N. benthamiana Leaves**

*Pseudomonas syringae* pv. *Tomato* DC3000 was cultured on King’s B (KB) medium containing 50 µg ml<sup>-1</sup> rifampicin. Overnight, log-phase cultures were grown to an optical density at OD<sub>600</sub> of 0.6–0.8 (OD 0.1 = 10<sup>8</sup> cfu ml<sup>-1</sup>) and diluted with 10-mM MgCl<sub>2</sub> to the concentrations of 10<sup>5</sup> CFU ml<sup>-1</sup> before inoculation. Control was performed with 10-mM MgCl<sub>2</sub>. The bacterial suspensions were infiltrated into the abaxial surface of a leaf using a 1-ml syringe without a needle. *Agrobacterium tumefaciens* LBA4404 transformant cells carrying *LasP*<sub>235</sub> and LTP protein cloned in pR101 vector was cultured overnight in LB medium with 50 µg ml<sup>-1</sup> of rifampicin and 50 µg ml<sup>-1</sup> kanamycin and resuspended in 10-mM MgCl<sub>2</sub>, 10-mM MES. The culture was diluted to an optical density of 0.5 (OD 600 nm). For the assay, the infected leaves of *N. benthamiana* plants were infiltrated with the *A. tumefaciens* suspension containing the LTP alone, LTP + *LasP*<sub>235</sub>, different mimics (Liu et al., 2013). The presented data were an average of three replicates, and two of them were of technical replicates.

**The qPCR Analysis**

The concentration, quality, and integrity of the DNA was analyzed using the Agilent 2100 bioanalyzer (Bio–Rad) and NanoDrop<sup>TM</sup> ND-1000 (Thermo Scientific). The qRT-PCR experiments were conducted using GoTaq qPCR Master Mix (Promega), *P. syringae* gene-specific primers (Psy-F ATGATCGGAGCAGCAAG; Psy-R GCT CTT GAG GCA AGC ACT), and *PR1b* (pathogenesis related protein): PR1F-CGAGAGGCGCAAGCTAATAC and PR1R: GCAAGAAATGACCCACCATCC gene from tomato genome was used as a standard to show that the variation in Ct values was due to infection and treatment with LTP or the mimics (Guilbault et al., 2016). The experiments were carried out with three biological replicates and each replicate divided into two technical replicates in a CFX-96 Bio–Rad thermocycler (Bio–Rad). Increasing temperature (0.5°C 10<sup>s</sup>−1) from 55 to 95°C was
used for melt curve analysis. The bacterial load corresponding to the CFU was calculated from standard curve. To determine the sensitivity of the qPCR assay, a culture of bacterial strain was diluted with sterile water to generate a 10-fold dilution series from $1 \times 10^8$ to $1 \times 10$ CFU·ml$^{-1}$. Each dilution ($1 \mu$l) of bacterial suspension was used as templates for quantifying bacterial load by direct qPCR without a DNA extraction step. The resulted Ct values were plotted against the corresponding CFU·ml$^{-1}$ value to generate a standard curve for the detection limit. Each dilution was analyzed in three replicates.

**Data Analysis**

For each of the investigated parameters, the experiments were conducted in triplicate with two technical replicates. All experimental data values were expressed as means of three measurements [± standard error (SE)]. The significance of the differences between the mean values were statistically evaluated by two-sided $t$-test at $p \leq 0.05$ using the Windows 2004/Microsoft Excel computer package for significance. The $K_m$ (Michaelis constant), $V_{\text{max}}$ were calculated using Lineweaver Burk plot. The $K_{\text{cat}} = V_{\text{max}}/E$, where $E$ is the total enzyme, i.e., free enzyme and enzyme bound to the substrate. $IC_{50} = (\text{Concentration of the effector protein} \times 50)/\%$ inhibition.

**Molecular Modeling**

**Prediction of Protein 3D Structures and Complexes**

The 3D structures of the two CLas proteins (LasP$_{235}$ and Effector 3) and the two citrus proteins (LTP and KTI) were predicted using I-TASSER (https://zhanglab.ccmb.med.umich.edu/I-TASSER/). We then used HADDOCK version 2.2 webserver to predict interaction interfaces of LasP$_{235}$-LTP and Kunitz-E3 complexes (http://milou.science.uu.nl/services/HADDOCK2_2/). The selected complexes of LasP$_{235}$-LTP and Kunitz-E3 were further refined using molecular dynamics (MD) simulations of these complexes in the presence of water.

**Protein-Water System Setup for MD Simulation**

Our simulations started with single protein (i.e., LTP, LasP$_{235}$, Kunitz or E3) in water. These systems contained 10,000 water molecules in a box of $6.9 \times 6.8 \times 7.1$ nm. To refine the models of LasP$_{235}$-LTP and Kunitz-E3 obtained from HADDOCK, we conducted MD simulations of these complexes in the presence of water. The protein–protein complex systems contain 30,000 water molecules in a box of $9.9 \times 9.9 \times 9.9$ nm with excess NaCl at 150 mM to mimic experimental conditions. For Kunitz-E3 complexes, we focus on model with Kunitz’s active loop in close contact with E3’s interface that contain either aspartic acid or glutamic acid residues or a large hydrophobic surface. For LasP$_{235}$-LTP complexes, we focus on model with LTP’s lipid entrance site B1 and B2 (see Supplementary Figure 3) in close contact with LasP$_{235}$. Following the MD simulation, the systems with stable complexes and adequate protein–protein pairwise residues interactions were then further validated by extended MD simulation.

** RESULTS**

** In vitro Protein Assay to Identify the Citrus Protein Targets of LasP$_{235}$ and Effector 3**

The LasP$_{235}$ identified in the prophage region of Las genome encoded a 123 amino-acid protein has an N-terminal nuclear localization signal (NLS) with no typical signal peptide (Hao et al., 2019). Effector 3 on the contrary has a predicted chloroplast targeting signal sequence (Pitino et al., 2016, 2018). The homology modeling predicted the presence of helical bundles in the structure of LasP$_{235}$ as shown in Supplementary Figure 1A. Note that the similar helical bundles are also present in AvrRps4, a P. syringae effector involved in plant immunity (Sohn et al., 2012). It is suggested that the helical effectors from bacteria may interact with multiple plant helical proteins via intermolecular
coiled–coil interactions (Chan et al., 2010; Goritschnig et al., 2016). These proteins may be located on the plasma membrane, in the cytosolic fluid or vacuole, and in the nucleus. The homology modeling also predicted two helix bundles in the structure of Effector 3 in addition to a disordered C-terminal segment (see Supplementary Figure 1B). The latter may make Effector 3 a promiscuous binding partner of several citrus proteins. In addition, due to the presence of chloroplast targeting signal, Effector 3 may be a potential CLas effector. Note that multiple chloroplast proteins are involved in ROS production and plant hormone signaling (Sowden et al., 2018), which may mediate cell death as an innate immune defense.

The steps in our target identification scheme are shown in Figure 1 (left). First, we expressed LasP235 and Effector 3 in E. coli with C-terminal His6-tags. The Effector 3 was expressed without the signal sequence. Both proteins were extracted from the inclusion body and re-folded. Second, the His-tagged LasP235 and Effector 3 were bound to TALON columns and were incubated with citrus protein extracts from the uninfected and CLas-infected Hamlin. Third, bound citrus protein targets were eluted from the column and identified by LC–MS/MS. Finally, the spectra from LC–MS/MS were searched against the Uniprot database with taxonomy set to C. sinensis. The highest ranked citrus proteins, in terms of the LC–MS/MS protein score (Koenig et al., 2008), were selected as putative targets of LasP235 and Effector 3. See Supplementary Tables 1A,B for all citrus targets of LasP235 and Effector 3 with high protein scores. Supplementary Table 1C lists the background targets as obtained by first incubating the TALON column with the protein extraction buffer and then elution by the citrus protein extract. Note that the non-specific targets with low protein scores were obtained by buffer elution. As shown in Figure 1 (right), the top-ranked citrus targets of LasP235 and Effector 3 show protein scores far greater than those listed for the non-specific targets in Supplementary Table 1C. A subset of these targets was further analyzed. The selected LasP235 targets are SOD (from infected citrus), LTP (from healthy citrus), Aspartyl Endopeptidase, (AP) and Glycosyl Hydrolase family 17, GH17 (from both healthy and infected citrus) whereas the Effector 3 targets are as follows: KTI and ALDH2 from both healthy and infected citrus, Elongation Factor Tu (Ef–Tu) from infected citrus, lectin, and 21 kDa seed protein-like (a functional homolog of KTI) from healthy citrus. As indicated, all target proteins listed in Figure 1 (right) are involved in citrus innate immunity. Although, it allows identification of both extracellular and intracellular targets of CLas effectors from infected and healthy citrus, our method in Figure 1 is likely to miss the citrus targets that are expressed at a low level.

**In planta Validation of the Citrus Protein Targets of LasP235 and Effector 3**

In planta validation is based on a split triple GFP assay, which has been successfully applied to monitor protein–protein interactions in yeast, human, and plant (Cabantous et al., 2013; Pedelacq et al., 2019). The assay relies on the principle that specially enhanced 11 stranded GFP can be split into GFP-1-9, GFP-10, and GFP-11 with none of the three split components showing fluorescence. However, the fluorescence is recovered when GFP-1-9, GFP-10, and GFP-11 are re-assembled. We constructed stable transgenic tobacco lines that overexpress LasP235 (or Effector 3) with a GFP-11 tag and the other a putative citrus target with a GFP-10 tag, were infiltrated in the GFP-1-9 transgenic tobacco. As shown in the experimental design of Figure 2A, we expect to observe (i) green fluorescence in the presence of a target–effector interaction and (ii) no fluorescence in the absence of interaction. In our assay, for negative controls (see Figure 2B), we confirmed the lack of interaction between Effector 3 and the targets for LasP235 (and the lack of interaction between LasP235 and the targets for Effector 3). Agrobacterium carrying enhanced GFP was used as a positive control. Note that the leaves in the negative control appear red in color due to chlorophyll autofluorescence. The fluorescence coming from chlorophyll molecules is a result of emission characteristics of both individual chlorophyll molecules and the fluorescence is observed at excitation and emission maxima of 685 and 720–730 nm. Figure 2B (top) shows the results of the split GFP assay monitoring the interaction of LasP235. Note the presence of green fluorescence at the infiltrated leaf sites for SOD, LTP, AP, and GH17, which were identified as putative targets of LasP235 as identified from our *in vitro* protein assay as described Figure 2A. The pattern of fluorescence is comparable to the infiltration of agrobacterium carrying enhanced GFP. Thus, the split GFP assay shows specific *in planta* interactions between LasP235 and citrus proteins (SOD, LTP, AP, and GH17). Figure 2B (bottom) shows the results of the split-GFP assay monitoring the interaction of Effector 3. The presence fluorescence at the filtrated sites indicates specific *in planta* interactions between Effector 3 and (KTI, ALDH2, lectin, and Ef–Tu) that were identified by the *in vitro* protein assay. Triple split GFP assay provides the following advantages (Serebriskii et al., 2000; Rajagopala, 2015) over other commonly used assays such as yeast-two hybrid system for monitoring protein–protein interaction: (i) It can be readily adapted to *in planta* systems; (ii) It limits false positives and negatives; (iii) Small GFP10 and GFP11 tags retain native effector–target interactions; (iv) Positive and negative controls can easily be incorporated for *in planta* measurement to improve the fidelity of the assay.

**The Two CLas Proteins Inhibit the Functions of Their Specific Citrus Targets In vitro Assays**

Three targets of LasP235, i.e., SOD, AP, and GH17, that are validated by *in planta* split GFP assay, are citrus PR or defense proteins with enzymatic activities. As described in the “Methods,” the citrus target proteins were expressed in *E. coli*, extracted from the inclusion body, and purified by affinity purification schemes. After purification, the proteins were re-folded. The identity of the proteins was confirmed by western blot analysis (Supplementary Figure 2) and Mass spec analysis (data are not shown). Therefore, before conducting *in vitro* inhibition assays,
FIGURE 1 | Identification of citrus target proteins of the CLas effectors. (Left) Outlines of the experimental steps. Step 1. CLas effectors were overexpressed in E. coli with His tag. Purified His6-tagged effectors (colored green) were incubated with protein extracts from healthy and infected Hamlin citrus. Specific citrus target protein (colored orange and magenta) bound to the CLas effectors. Step 2. Effector-target complexes were TALON on Agarose beads and non-target citrus proteins were washed away. Step 3. The specific effector-target complexes were eluted. Step 4. The citrus target proteins from the eluted complexes were identified by LC–MS/MS. (Right) Selected citrus protein targets of the CLas effectors, LasP235 and Effector 3. The citrus targets were chosen on the basis of their high protein scores. The target proteins were selected both from healthy and CLas-infected protein extracts. GenBank sequence IDs and putative functions (based on the literature data) of the citrus targets are listed.

it was necessary to determine the enzymatic activities of the recombinant enzymes to confirm that they retained the native fold and function. We then determined the inhibitory activity of LasP235 on them by measuring IC50 (the concentration required for 50% reduction in enzymatic activity). The SOD, which is unique to plants, prevents damage caused by the ROS burst upon pathogen infection (Miller, 2012). While it facilitates the direct killing of the pathogen and induction of plant defense genes, excessive ROS is damaging to the plant. The SOD, produced in mitochondrion, peroxisome, and chloroplast, converts oxygen radical to molecular oxygen and hydrogen peroxide. The latter, also potentially phytotoxic, is subsequently converted by plant catalase into molecular oxygen and water. The SOD is also involved in regulating ROS signaling leading to the induction of defense genes (Wang et al., 2018). As shown in Table 1, LasP235 inhibits the activity of the citrus SOD. The Citrus AP belongs to the A1 family of atypical aspartate proteases, primarily located in apoplast and chloroplast. It has been shown that an atypical aspartate protease, expressed in the apoplast, confers constitutive disease resistance 1 (CDR1) in Arabidopsis probably by producing a peptide ligand through cleavage and subsequent induction of SA signaling and expression of PR genes (Varghese et al., 1994; Simões et al., 2007). The results of enzyme assay show that LasP235 inhibits the activity of the citrus AP. The GH17, a citrus (β1-3) glucanase, is another direct interactor of LasP235. Typically, GH17 glucanases are known to provide disease resistance against fungi by hydrolyzing fungal chitins (Hrmova and Fincher, 2001). However, GH17 also has a role in immune defense in general in that it regulates the formation of callose (a β1-3 glucan polysaccharide), which is an essential component of papillae, an ultrastructure formed at the site of pathogen penetration. Apart from callose, the papillae also contain ROS and antimicrobial peptide thionin and thus provide the first line of defense against pathogen invasion. In papillae-mediated immunity, callose may be involved in two different mechanisms of plant defense against pathogens. First, the callose deposition in papillae may block pathogen spread. Second, the hydrolyzed products of callose by GH17 may serve as ligands for the PRRs and may induce SA signaling leading to plant immune defense. Thus, the GH17-mediated hydrolysis of callose may either support pathogen spread or induce SA signaling. As evident from Table 1, LasP235 inhibits the glucanase activity of the citrus GH17. Therefore, it may induce SA signaling and help CLas to suppress citrus immune defense. The citrus
FIGURE 2 | In planta validation of the selected citrus protein targets of \( \text{LasP}_{235} \) and Effector 3 by triple split GFP assay. (A), left] A schematic representation of the principle of the triple split GFP assay. Green fluorescence is observed when the Effector (cyan) linked to GFP10 (yellow) interacts with the target (blue) linked to GFP11 (orange) and the effector-target complex complements with GFP1-9. There is no fluorescence in the absence of an interaction. (A), right] The presence of fluorescence when agrobacterium carrying enhanced GFP is infiltrated on the leaves of transgenic tobacco expressing GFP1-9 is used as a positive control. Absence of fluorescence when the leaves of transgenic tobacco expressing GFP1-9 co-infiltrated with \( \text{LasP}_{235} \) and the interactors of Effector 3 in one-half of the leaf (or Effector 3 and the interactors of \( \text{LasP}_{235} \) in the other half of the leaf) served as the negative controls. Note the absence of fluorescence. Under the 488 nm excitation filter (blue) and colored glass 520 nm long pass filter, the chlorophyll background appears as red and fluorescence at the site of co-infiltration of the effector and its target appears as greenish yellow spots. (B) Complex formation when \( \text{LasP}_{235} \) is co-infiltrated with SOD, LTP, AP, and GH17 in the leaves of GFP1-9 transgenic tobacco (top panel) and when Effector 3 is co-infiltrated with Effector 3 and KTI, ALDH, Ef–Tu, and Lectin (bottom panel).

LTP is the non-enzyme direct interactor of \( \text{LasP}_{235} \). The plant LTPs possess (i) lipid binding property, which is critical to lipid homeostasis and membrane dynamics and (ii) bactericidal activity as a component of immune defense (Liu et al., 2015; Finkina et al., 2016; Salminen et al., 2016; Shenkarev et al., 2017). Table 1 shows that \( \text{LasP}_{235} \) can block both lipid-binding

Table 1 | SH3-0 Binding Activity of \( \text{LasP}_{235} \) and Effector 3

| Protein | SH3-0 Binding Activity |
|---------|------------------------|
| \( \text{LasP}_{235} \) | Weak |
| Effector 3 | Strong |

Table 1 shows that \( \text{LasP}_{235} \) can block both lipid-binding
The citrus AP, SOD, GH17, and ALDH were overexpressed in and purified from E. coli and enzymatic assays were performed on them following the protocols described in Experimental Procedures section.

| Citrus targets | K_{cat} (s^{-1}) | K_{m} (mM) | IC50 | Substrate/ligand concentration (mM) | Target concentration (mM) | MIC (µM) | MIC (µM) | MIC (µM) | MIC (µM) |
|----------------|-----------------|------------|------|-------------------------------|--------------------------|-----------|-----------|-----------|-----------|
| P235           | AP              | 277.2      | 315.8| 6.30                          | 10                       | 20        | 20        | 20        | 20        |
|                | SOD             | 12.45      | 42.3 | 4.37                          | 2                        | 4         |           |           |           |
|                | GH17            | 0.003      | 0.042| 1.95                          | 1                        | 25        |           |           |           |
|                | LTP             | 1.43       |      |                               |                          |           |           |           |           |
| Effector 3     | KTI             | 5.60       |      |                               |                          |           |           |           |           |
|                | ALDH            | 0.094      | 2.131| 0.86                          | 4                        | 10        |           |           |           |

K_{cat} and K_{m} are given for the citrus AP, SOD, GH17, and ALDH. The IC_{50} (50% reduction in catalytic activity) by the effector is provided as the ratio of the substrate concentration.

Lectin or Ef–Tu had negligible effect on the ROS level. The co-infiltration of Effector 3 plus lectin or Ef–Tu had very little effect on the ROS level induced by Effector 3 alone. However, combination of lectin and Ef–Tu was able to reduce the ROS level induced by Effector 3. In this regard, it is important to note that some bacteria, such as Porphyromonas gingivalis, Mycobacterium tuberculosis, Helicobacter pylori, and Bacillus anthracis, utilize ROS to support their growth and to establish infection (Paiva and Bozza, 2014) whereas plant lectin and Ef–Tu tend to inhibit ROS production or ROS-mediated signaling (Wang and Bouwmeester, 2017). It appears that pathogenic CLas may use Effector 3 to maintain ROS level that is beneficial to pathogen growth and infection by inhibiting anti-ROS citrus lectins and Ef–Tu. The PQ was also used to induce PCD via ROS in tobacco. The PCD was monitored by electrolyte leakage (Kacprzyk et al., 2016), which was set to 100% as induced by agrobacterium carrying an empty vector plus PQ. Infiltration of the agrobacterium carrying Effector 3 induced ~50% electrolyte leakage, which, as shown in Figure 3B (Right), was reduced on infiltration of agrobacterium carrying citrus KTI. The co-infiltration of Agrobacteria carrying citrus KTI and Effector 3 elevated PCD thereby confirming that Effector 3 is an inhibitor of the citrus KTI.

**To Predict and Validate the Molecular Mechanism of Effector–Target Inhibitory Interactions**

We performed all-atom MD simulations (Li et al., 2018) to predict the interactions that stabilize the (inhibitory CLas effector–citrus protein target) complexes. Initially, we focused on the bactericidal effect of LTP. As described in Materials and Methods section, we first obtained an optimized homology-based model of the citrus LTP as shown in Supplementary Figure 1C. Then, we performed MD simulations in (water: lipid) bilayer for 10 µs. As described in the Supplementary Figure 4A, MD simulations revealed that the LTP helices h2, h3, h4, and the C-terminal loop were involved in interaction with the lipid bilayer defining membrane attachment, which is the first step in the
bactericidal activity. The interactions of the positively charge arginine residues R21, R32, R39, R44, R71, and R89 (shown in blue in Figure 4B) with the negatively charged lipid polar heads appear to be extremely critical for the LTP membrane attachment. To study the interaction of \( \text{LasP}_{235} \) with LTP, we docked the homology based \( \text{LasP}_{235} \) model to the optimized LTP model. We then performed MD simulations of the LTP-\( \text{LasP}_{235} \) complex in aqueous environment for 6 \( \mu \)S to determine which mode of \( \text{LasP}_{235} \) binding may block the LTP attachment to the lipid bilayer as discussed in Supplementary Figures 4A,B. One mode of \( \text{LasP}_{235} \) (magenta) interaction, shown in Figure 4A (left), involves the LTP (cyan) helices h2, h3, and h4 and the C-terminal loop resulting in partial blocking of the B1 LTP site by \( \text{LasP}_{235} \). The prominent pair-wise contacts between \( \text{LasP}_{235} \)
FIGURE 4 | Prediction and validation of (citrus target–CLas effector) interaction at the contact interface. Molecular modeling is performed to predict the pairwise interactions based on which mimics are designed to displace the effector from the (citrus target–CLas effector) complex. Finally, experiments are performed to determine if the mimic, indeed, displaces the effector from the complex and if so, our prediction of the pairwise interactions are validated. (A) Two possible modes of

(Continued)
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FIGURE 4 | Interactions between LasP₂₅₅ (magenta ribbon) and LTP (cyan ribbon) which block the membrane attachment of LTP thereby inhibiting the bactericidal activity. The disulfide bridges are shown as yellow sticks. The LTP helices (h2, h3, and h4) and the C-terminal segment are predicted to be involved in one mode of interaction. The LTP helices (h1, h2, and h3) are involved in the other mode of interaction. Important residues in the pairwise contacts are shown: LasP₂₅₅ residues are labeled black whereas the LTP residues are labeled red. Basic, acidic, neutral, and acidic residues are, respectively, as blue, red, green, and gray sticks. (A) Amino acid sequences of Mimic 1 and Mimic 2 at the bottom. Bacterial (P. syringae pv. tobaci) clearance in tobacco by the two mimics in the presence and absence of LasP₂₅₅. Note that an excess of the mimics is needed for significant bacterial clearance. Mimic 1 is a better bactericidal than Mimic 2. LasP₂₅₅ is an inhibitor of Mimic 1 or Mimic 2. (B) Two models of interactions between Effector 3 (magenta ribbon) and KTI (cyan ribbon with the reactive loop in red). Both models show interactions with the KTI reactive loop as a prominent mode of inhibition. The predicted pairwise interactions are shown. The residues from Effector 3 are shown as gray sticks and labeled black whereas the residues from KTI are shown as blue sticks and label red. *Indicates statistical significance at p ≤ 0.05.

DISCUSSION

Bacterial effectors are known to inhibit plant innate immune signaling networks mediated by PTI, ETI, and plant SA, JA, and ET hormones. The end products of PTI, ETI, and plant hormone signaling are the PR or immune defense proteins that either clear the invading the pathogen or block infection. Typically, each immune defense protein is induced at a low level and a single protein; therefore, can neither completely clear the pathogen nor can it totally block the infection. Interestingly, simultaneous induction of multiple immune defense proteins (albeit at low levels) can lead to effective clearance of the invading bacteria and blocking of infection caused by them. However, the multiple effectors from a pathogenic bacterium such as CLas can suppress multiple signaling steps to support bacterial growth and infection. Here, we report the role of two CLas effectors, LasP₂₅₅ and Effector 3, in HLB pathogenesis. The each of them may directly target and inhibit more than one citrus innate immune defense proteins involved in bactericidal and/or disease-blocking activity. For example, LasP₂₅₅ can inhibit the citrus targets (SOD, AP, GH₁₇, and LTP) whereas Effector 3 can inhibit citrus targets (KTI, ALDH, Lectin, and Ef-Tu). Although, as shown here, a bacterial effector may target several plant proteins, inhibitions of all targets may not be equally important for bacterial pathogenesis. A direct evaluation of the importance of each (plant protein–bacterial effector) interaction is traditionally obtained by knockout of a specific bacterial effector. Since CLas is not culturable, it is not possible to conduct gene knockout experiments. However, the inhibitory activities of a CLas effector against different citrus targets reveal qualitatively the relative importance of different inhibitory (CLas effector–citrus target) interactions in HLB pathogenesis. For example, as shown in Table 1, LasP₂₅₅ is a potent inhibitor of LTP because at equimolar concentration it can completely block the bactericidal activity of LTP. Thus, LasP₂₅₅ may play an important role in HLB pathogenesis. Note that, relatively low IC₅₀ values (within 1 to 6) in Table 1, argue that the corresponding inhibitory interactions may be relevant in HLB pathogenesis. Figure 5 schematically summarizes the combined effect of the inhibitory interactions of LasP₂₅₅ and Effector 3 on their citrus targets as determined from our in vitro and in
LasP235 and Effector 3: elevation of ROS level, premature PCD, and inhibition of CLas clearance.

We analyzed the detailed interactions at the contact interfaces of the (LasP235-LTP) and (Effector 3-KTI) complexes. The molecular modeling and mutational analysis revealed the predominant mechanism of LTP inhibition by LasP235. We were able to design Mimic 1 (derived from LTP with specific amino acid substitutions) that showed intrinsic bactericidal activity and exhibited LasP235 inhibitory activity. The Mimic 1 can be further modified to increase its LasP235 inhibitory and bactericidal activity. We have also obtained two modes of inhibition in which Effector 3 may block the reactive loop of the citrus KTI. We have not yet completed in planta experiments to determine whether one of the two modes of inhibition or both may be important. Nonetheless, the citrus KTI as the target of inhibition by a CLas effector is an interesting observation since such inhibition may cause premature PCD, which may be beneficial to CLas in causing infection (Randow et al., 2013).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

GG contributed to project planning, experimental work, data analysis, and writing of the manuscript. SB performed the majority of in vitro and in planta studies, data analysis, and writing of the manuscript. LH performed the MD simulations and analyses. HN assisted in the split GFP assays. RR performed expression of some recombinant proteins. JV performed some MIC assays. ES supervised the work of GM and QS on citrus infection by CLas and protein extraction from healthy and infected citrus and the work of GH and SZ toward generating transgenic tobacco expressing GFP1-9. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.869178/full#supplementary-material

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