Drug Repurposing: Tolfenamic Acid Inactivates PrbP, a Transcriptional Accessory Protein in *Liberibacter asiaticus*

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CLIBASIA_01510, PrbP, is a predicted RNA polymerase binding protein in *Liberibacter asiaticus*. PrbP was found to regulate expression of a small subset of ribosomal genes through interactions with the β-subunit of the RNA polymerase and a short, specific sequence on the promoter region. Molecular screening assays were performed to identify small molecules that interact with PrbP *in vitro*. Chemical hits were analyzed for therapeutic efficacy against *L. asiaticus* via an infected leaf assay, where the transcriptional activity of *L. asiaticus* was found to decrease significantly after exposure to tolfenamic acid. Similarly, tolfenamic acid was found to inhibit *L. asiaticus* infection in highly symptomatic citrus seedlings. Our results indicate that PrbP is an important transcriptional regulator for survival of *L. asiaticus in planta*, and the chemicals identified by molecular screening assays could be used as a therapeutic treatment for huanglongbing disease.

**Keywords:** transcriptional accessory protein, *Liberibacter asiaticus*, tolfenamic acid, antimicrobial, citrus

**INTRODUCTION**

*Liberibacter asiaticus* is the prevalent causative agent of huanglongbing (HLB; citrus greening), the most devastating citrus disease worldwide. Since its emergence in the early 1900's in China, *L. asiaticus* has been found in nearly every citrus producing region across the globe, with devastating consequences (Bove et al., 2006). In the United States, the economic impact of HLB has already been felt throughout the Florida citrus industry, where estimated losses have exceeded $9 billion since the pathogen was first detected in 2005; and the California citrus industry is preparing for similar devastation, as this pathogen continues to spread across the west coast (Gottwald, 2010).

*Liberibacter asiaticus* is a phloem-limited pathogen with uneven distribution throughout the plant. It is transmitted by the Asian citrus psyllid *Diaphorina citri* and it behaves as intracellular plant pathogen and as an insect symbiont. These characteristics has made the control and eradication of *L. asiaticus* a scientific challenge with no precedents. The lack of stable culturing conditions for *L. asiaticus*, in a laboratory setting, has severely hampered progress toward understanding the physiology and adaptive strategies of this citrus pathogen. Genome analyses have revealed a drastic reduction of the genetic information encoded by the *L. asiaticus* genome,
suggesting this microorganism is highly adapted to life within its host (Duan et al., 2009). One of the functional protein clusters suffering the highest negative selection were transcription factors, which represent only 2% of the total proteins encoded in the genome (Duan et al., 2009; Pagliai et al., 2014). Interestingly, the small group of transcription factors preserved in \( L. \text{asiaticus} \) includes the gene systematically annotated as \( \text{CLIBASIA}_01510 \) (here named \( \text{PrbP} \)). \( \text{PrbP} \) is a member of the \( \text{CarD}_\text{CdnL} \_\text{TRCF} \) superfamily (CDD cl00588). \( \text{PrbP} \) shares low sequence identity with the transcription factor \( \text{CdnL} \) in \( \text{Myxococcus xanthus} \) and with \( \text{CarD} \) in the \( \text{Mycobacteriaceae} \) family (García-Moreno et al., 2010). Members of this protein family have been linked to pathogenesis, persistence, cell viability, and resistance to both antibiotics and environmental stress (Stallings et al., 2009; Stallings and Glickman, 2011; Weiss et al., 2012).

In absence of laboratory culturing conditions for \( L. \text{asiaticus} \), traditional methods for testing chemicals as antimicrobials \( \text{in vitro} \) cannot be applied. In this study, we explored the possibility of identifying specific ligands \( \text{in vitro} \), that affect the activity of \( \text{PrbP in vivo} \), as a means to disrupt gene expression, and ultimately decrease viability of \( L. \text{asiaticus} \) in the citrus host. Only three eukaryotes, \( \text{Spinacia oleracea} \), \( \text{Beta vulgaris} \), and \( \text{Drosophila elegans} \), were found to carry \( \text{PrbP} \) homologs (74, 72, and 46% identity, respectively). The absence of a \( \text{PrbP} \) homolog in citrus renders \( \text{PrbP} \) a unique target for therapeutics. Furthermore, the use of ligands that inactivate specific pleiotropic transcription factors, enables the selective elimination of target species in heterogeneous populations. Taken together, \( \text{PrbP} \) represents an excellent therapeutic target for the design of antimicrobial strategies against \( L. \text{asiaticus} \).

**MATERIALS AND METHODS**

**Bacterial Strains and Growth Conditions**

\( E. \text{coli} \) strains were grown at 37°C under aerobic conditions in Luria-Bertani medium (LB) (Difco) or on LB agar plates. \( E. \text{coli} \) strains DH5a (Invitrogen, Carlsbad, CA), TOP 10 (Invitrogen), and XL1-Blue (Stratagene, La Jolla, CA) were used to propagate the plasmids for protein purification, point mutations, and the two-hybrid system (the bacterial strains and plasmids used in this study are listed in Table 1). \( E. \text{coli} \) strain BL21-Rosetta(DE3) (Novagen, Gibbstown, NJ) was used for overexpression and protein purification. When required, the medium was supplemented with ampicillin (100 µg/ml), tetracycline (10 µg/ml), kanamycin (50 µg/ml), or chloramphenicol (25 µg/ml). All antibiotics and chemicals were purchased from Sigma (St. Louis, MO).

\( \text{Liberibacter crescens} \) BT-1 was cultured at 25°C with moderate aeration (150 RPM), in modified BM7 media (Leonard et al., 2012) containing 1% \( \text{Brain Heart Infusion} \) (Difco Laboratories, Detroit, MI), 15% Fetal Bovine Serum (Sigma, St. Louis, MO), 30% \( \text{TMN-FH insect medium} \) (Sigma), \( \text{α-Ketoglutaric acid} \) (2 mg/ml), ACES (10 mg/ml), and potassium hydroxide (3.75 mg/ml), at pH 6.9. For determinations of the antimicrobial activity of metronidazole, dimetridazole, ronidazole, and tolfenamic acid, each chemical was added to the liquid media at increasing concentrations (1–500 µM). The optical density was determined at 600 nm every 24 h during 5 days.

**DNA Manipulations and Gene Cloning**

Standard methods were used for chromosomal DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, ligation, and transformation (Pagliai et al., 2010). Plasmids were isolated using the QiAprep\textsuperscript® Spin Miniprep Kit (Qiagen, Valencia, CA), and PCR products were purified using Qiaquick\textsuperscript® Purification Kits (Qiagen). All of the primers used in this study are described in Table 2. For protein expression and purification, the \( \text{prbP (CLIBASIA}_01510 \) gene was amplified by PCR, using total DNA extractions from \( L. \text{asiaticus} \) infected tissue, and cloned into the p15TV-L plasmid as described previously (Pagliai et al., 2010).

**Two-Hybrid System**

The two-hybrid system previously described by Borloo et al. (2007) was used (Borloo et al., 2007). Proteins of interest were fused to complementing \( β\text{-galactosidase} \) truncations (\( Δω \) and \( Δα \)), where the resulting level of complemented \( β\text{-galactosidase} \) activity corresponds directly to the level of interaction between the proteins. Proteins of interests were cloned into vectors pB2H\( Δα \) and pB2H\( Δω \) using the NotI and BamHI restriction sites. All subcloning steps were performed in \( E. \text{coli} \) XL-1 Blue (Stratagene, La Jolla, CA). Fusion proteins in pB2H\( Δα \) and pB2H\( Δω \) were transformed by heat shock and subsequently co-expressed in \( E. \text{coli} \) TOP10. Empty vectors were used as a control to determine baseline activity. The minimal inhibitory concentration (MIC) was determined for each chemical in \( E. \text{coli} \) (Table 3). The highest concentration that did not adversely affect the base levels in the controls was used for subsequent \( \text{in vivo} \) assays.

**β-Galactosidase Assays**

\( E. \text{coli} \) cells were grown at 37°C in LB medium until reaching OD600 of 0.8 (late exponential phase). Cells were collected and lysed in Z-buffer (60 mM \( \text{Na}_2\text{HPO}_4 \), 40 mM \( \text{NaH}_2\text{PO}_4 \), 10 mM \( \text{KCl} \), 1 mM \( \text{MgSO}_4 \), 50 mM \( \text{β-mercaptoethanol} \); Miller, 1972). \( β\text{-galactosidase} \) activity was assayed by following the catalytic hydrolysis of chlorophenol red-\( β\text{-D-galactopyranoside} \) (Sigma-Aldrich). The absorbance at 570 nm was read continuously using a Synergy HT 96-well plate reader (BioTek, Winooski, VT). \( β\text{-galactosidase} \) activity, expressed as arbitrary units (AU), was calculated using the slope of absorbance curve normalized with the initial cell density. Preliminary \( β\text{-galactosidase} \) assays were performed with strains LB01 and LB02, in the absence and presence (0–1 mM) of each chemical to determine baseline activity. Each assay was performed in triplicates.

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**Abbreviations:** EMSA, electrophoretic mobility shift assay.
Protein Purification

Protein purification was performed as previously described (Lorca et al., 2007). Briefly, PrbP was cloned into vector p15TV-L. The His-tagged fusion protein was then overexpressed in E. coli BL21-Rosetta(DE3) (Novagen). Cells were grown in LB broth at 37°C, to an OD600 of 0.6. Expression was induced with 0.5 mM isopropyl -thio-β-D-galactosidase (IPTG). After induction, the cells were incubated at 17°C for 16 h. The cells were harvested and resuspended in binding buffer (500 mM NaCl, 5% glycerol, 50 mM HEPES, 5 mM imidazol, pH 7.5) with Roche EDTA-free protease inhibitor cocktail (Roche Applied Science, Germany). Phenylmethylsulfonyl fluoride (0.5 mM) and Tris(2-carboxyethyl)phosphate hydrochloride (0.5 mM) were added to the cells immediately before lysing. Cells were lysed using a French press. The lysates were clarified by centrifugation (30 min at 17,000 × g) and applied to a metal chelate affinity column charged with nickel. The column was washed extensively with binding buffer containing 20 mM imidazole, and the proteins were subsequently eluted from the column with elution buffer (binding buffer containing 250 mM imidazole). The purified proteins were dialyzed against 10 mM HEPES (pH 7.5), 500 mM NaCl, 2.5% glycerol, 0.5 mM TCEP, and stored at −80°C. The identity of the purified proteins was confirmed by Mass Spectrometry (as a service in the Interdisciplinary Center for Biotechnology Research, University of Florida) from protein bands isolated from SDS-PAGE gels.

Electrophoretic Mobility Shift Assays (EMSA)

Gel shift assays for PrbP were performed using aliquots of protein purified and concentrated according to the procedures described above. Fragments of the rplK, prbP, rpsi, dnaK, and radA promoter regions were generated by PCR using pre-labeled 5′-biotin primers (Table 2). PCR products were purified using QIAquick spin columns (Qiagen). EMSA reaction mixtures (20 μL) contained 1 ng of 5′ biotin-labeled DNA probe, 12.5 ng/μL of both Poly(dI-dC)-Poly(dA-dT) nonspecific competitor DNAs, 10 mM Heps pH 7.5, 150 mM NaCl, 5% glycerol, purified PrbP protein (0−7 μM), and ligand (0−2 mM) as indicated. Competition assays were carried out using unlabeled fragments of the promoter regions generated by PCR. After incubation at 37°C for 20 min, samples were separated on 6% acrylamide-bisacrylamide non-denaturing gels, in ice cold Tris borate-EDTA buffer, pH 8.3 (TBE). Electrophoresis was performed on ice at 100 V for 2 h. DNA was transferred to a Hybond-N+ membrane (GE Healthcare, Pittsburgh, PA) with a Semi-Dry electroblotter (Fisher Scientific, Pittsburgh, PA) at 250 mA for 45 min. Transferred DNA was cross-linked to the membrane using

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**Table 1 | Bacterial strains and plasmids used in this study.**

| Strain or plasmid | Genotype or description | References |
|-------------------|------------------------|------------|
| **STRAINS**       |                        |            |
| DH5α              | F− Δ(lacZYA-argF)U169 recA1 endA1 hsdRI7 (rK2 mK2) phoA supE44 4 thi-1 gyrA96 relA1 | Invitrogen |
| BL21-Rosetta(DE3) | F− ompT hsdS D (rK2 mK2) gal dcm (DE3) pRARE | Novagen |
| JM109             | e44 (McrA−) recA1 endA1 gyrA96 thi-1 hsdRI7 (rK2, mK2) supE44 relA1 Δ(lac-proAB) [F’ traD36 proAB lacIq ZΔM15] | Promega |
| TOP10             | F− mcrA Δ(mrshsdRM5-mcriBC) ΔlacZ74 nupG recA1 araD139 Δ ara-leu7697 galE15 galK16 rpsL [Str−] endA1 λ− | Invitrogen |
| LB01              | TOP10 carrying empty pB2HΔω and pB2HΔω; Ampβ; Cmβ | This work |
| LB02              | TOP10 carrying pB2HΔω+prbP and pB2HΔω+rhoB21-150; Ampβ; Cmβ | This work |
| LB03              | TOP10 carrying pB2HΔω+rhoB21-150 and pB2HΔω+rhoB; Ampβ; Cmβ | This work |
| LB04              | TOP10 carrying pB2HΔω+prbP and pB2HΔω+rhoB; Ampβ; Cmβ | This work |
| LB05              | TOP10 carrying pB2HΔω+rhoB21-150; Ampβ; Cmβ | This work |
| L. crescens BT-1  | Wild type L. crescens strain BT-1 | CP003789 |
| **PLASMIDS**      |                        |            |
| p15TV-L           | Expression vector, adds 6X His tag, TEV cleavage site, Ampβ | EF456736 |
| p15TV-PrbP        | prbP gene from L. asiacus cloned in the BsrRI site of p15TV-L | This work |
| pB2HΔω            | pACYCDuet-1αc with the E. coli β-galactosidase fragment lacking the sequence for amino acids 11–41 (Δω) cloned in the BamHI-Ncol site; Cmβ | Borloo et al., 2007 |
| pB2HΔω+prbP       | pETDuet-1ΔSphIIαc with the E. coli β-galactosidase fragment lacking the sequence for amino acids 789–1023 (Δω) cloned in the BamHI–Ncol site; Ampβ | Borloo et al., 2007 |
| pB2HΔω+rhoB21-150 | pB2HΔω with a portion of the rpoB gene (encoding residues 21–150) from L. asiacus cloned in the NotII–BamHI site; Cmβ | This work |
| pB2HΔω+rhoB       | pB2HΔω with the prbP gene from L. asiacus cloned in the NotII–BamHI site; Ampβ | This work |
| pB2HΔω+rhoB21-150 | pB2HΔω with a portion of the rpoB gene (encoding residues 21–150) from L. asiacus cloned in the NotII–BamHI site; Ampβ | This work |

KnR, AmpR, CmR, and Tef indicate resistant to kanamycin, ampicillin, chloramphenicol, and tetracycline, respectively.
### TABLE 2 | Oligonucleotides used in this study.

| Primer              | Sequence (5′ → 3′)                     |
|---------------------|----------------------------------------|
| **qRT-PCR**         |                                        |
| CLIBASIA_01510-Fw   | CTGCCCATGAGTAAGGAATATTAC               |
| CLIBASIA_01510-Rv   | ATCTGTGCTCTGGTCAATGCAATTTAA           |
| CLIBASIA_00120-Fw   | TGGAGCGTGAATGGGCAAAA                   |
| CLIBASIA_00120-Rv   | CCAACGGAAAGATCAGATTTCTCCTTA           |
| CLIBASIA_r05785-Fw  | TCGAGCGCAGTATCCGCAATACG               |
| CLIBASIA_r05785-Rv  | GCGTTATCCGTTGAAAGAAGTAGA              |
| CLIBASIA_00130-Fw   | TCGAGATCCTAAACCTCTCTGGT               |
| CLIBASIA_00130-Rv   | ATACGCCCCCATATCTTGAGCATC              |
| CLIBASIA_00325-Fw   | TATCCCAATGGTCTGGTCAGA                 |
| CLIBASIA_00325-Rv   | GACCCGGTGCATAAGCATT                  |
| CLIBASIA_00735-Fw   | CGATCGAACGAAAATCTGACT                 |
| CLIBASIA_00735-Rv   | GGAAGGAAACCGACTCTATCTG               |
| COX-Fw              | GTATGCGACGTCGCACTTCGAGA               |
| COX-Rv              | GCGAAAACGTCGATTGACCTGTT              |
| 18S-Fw              | GCTTAGGCGAAGGAAGTTTG                 |
| 18S-Rv              | TCTATCCCATACAGATGAA                  |
| **DNASE I FOOTPRINT** |                                       |
| CLIB_00130_Fw_Fam   | CTGATGTCGCCGTTGCTCTC                 |
| CLIB_00130_Rv_Vic   | TGCAGAACCCCGACTCTATG                 |
| **EMSA**            |                                        |
| EMSA_CLIB_00130_Ext_Fw | CTGTTTTCTCAGAGTGTTG                  |
| EMSA_CLIB_00130_Ext_Rv | CCGCATAAAACGCCTCTAA                   |
| EMSA_CLIB_00130_Fw   | CTGATGTCGCCGTTGCTCTC                 |
| EMSA_CLIB_00130_Rv_Bio | TGCAGAACCCCGACTCTATG                 |
| EMSA_Cold_CLIB_00130_Rv | TGCAGAACCCCGACTCTATG                 |
| EMSA_CLIB_01510_Fw_Bio | TCTAACCCCTGTGCGATC                   |
| EMSA_CLIB_01510_Rv   | TGAAGAGTTATCCCTCCCTTAATCC            |
| EMSA_Cold_CLIB_01510_Fw | TCTAACCCCTGTGCGATC                   |
| EMSA_CLIB_RadA_Ext_Fw | GCTCTGTGCGTTGCGACATC                 |
| EMSA_CLIB_RadA_Ext_Rv | CAGAAGCAAGGGCTACATCA                 |
| EMSA_CLIB_RadA_Fw    | TCTATGTCGCCGTTGCTCTG                 |
| EMSA_CLIB_RadA_Rv_Bio | GGTATAGCAGTATCGGCGTG                  |
| EMSA_Cold_CLIB_RadA_Rv | GGTATAGCAGTATCGGCGTG                  |
| EMSA_CLIB_DnaK_Ext_Fw | TGCTGCTGATCAGTCAAC                   |
| EMSA_CLIB_DnaK_Ext_Rv | ACTGTTTCTCGTGCTCCGT                  |
| EMSA_CLIB_DnaK_Fw    | AATTCTCTTGCATCAGCC                   |
| EMSA_CLIB_DnaK_Rv_Bio | TCAATATGACGCAGCATGAA                 |
| EMSA_CLIB_RpsJ_Ext_Fw | TGCTGCTGCGATTCAA                     |
| EMSA_CLIB_RpsJ_Ext_Rv | CCGATATCGCGCTAGTCC                   |
| EMSA_CLIB_RpsJ_Fw    | GATGCGAGCCCAAATACGACT                |
| EMSA_CLIB_RpsJ_Rv_Bio | GGAAGCTAATCGCGAACCC                   |
| EMSA_CLIB_r05785_Fw  | AGAGAGAAAGGGAGAGCTGGA                |
| EMSA_CLIB_r05785_Rv  | CCAATGCTGATCCGCGAAGA                 |
| **SITE DIRECTED MUTAGENESIS** |                                      |
| rplKM1_Fw           | GTTTTTCTTATATAGTCGCTGGTGGTTTTTAG      |
| rplKM1_Rv           | CTTTTAAAAAACACCGCAATATACAAAAACAC     |
| rplKM2_Fw           | GGTGTTTTATATTAGTAGTTGGTGGTTTTTAG     |
| rplKM2_Rv           | CTAATTTAAAAAACACCAATATACAAAAACAC     |
| rplKM3_Fw           | GTTTTTATATAAGGCGCTGGGTTTTTAGAAAGG    |

(Continued)
### Table 2 | Continued

| Primer      | Sequence (5’ → 3’)                                                                 |
|-------------|-----------------------------------------------------------------------------------|
| rplKM3_Rv   | CCTTTTAAACACACGGCGTACTATATAAAAC                                                   |
| rplKM4_Fw   | GTTTTATAGTTAGTTATGGTTTTTAAGGCTAGG                                                 |
| rplKM4_Rv   | CCGAGGCTTTAAGGCGTACTATATAAAAC                                                   |
| rplKM5_Fw   | GCTTTTAAAGGCGTACTATATAAAAC                                                       |
| rplKM5_Rv   | CCGAGGCTTTAAGGCGTACTATATAAAAC                                                       |
| rplKM6_Fw   | GCTTTTAAAGGCGTACTATATAAAAC                                                       |
| rplKM7_Fw   | GCTTTTAAAGGCGTACTATATAAAAC                                                       |
| rplKM7_Rv   | GCTTTTAAAGGCGTACTATATAAAAC                                                       |
| rplKM8_Fw   | GCTTTTAAAGGCGTACTATATAAAAC                                                       |
| rplKM8_Rv   | GCTTTTAAAGGCGTACTATATAAAAC                                                       |
| rplKM9_Fw   | GCTTTTAAAGGCGTACTATATAAAAC                                                       |
| rplKM9_Rv   | GCTTTTAAAGGCGTACTATATAAAAC                                                       |
| rplKM10_Fw  | GCTTTTAAAGGCGTACTATATAAAAC                                                       |
| rplKM10_Rv  | GCTTTTAAAGGCGTACTATATAAAAC                                                       |
| rplKM11_Fw  | GCTTTTAAAGGCGTACTATATAAAAC                                                       |
| rplKM11_Rv  | GCTTTTAAAGGCGTACTATATAAAAC                                                       |
| rplKM12_Fw  | GCTTTTAAAGGCGTACTATATAAAAC                                                       |
| rplKM12_Rv  | GCTTTTAAAGGCGTACTATATAAAAC                                                       |

#### TWO-HYBRID CONSTRUCTS

| prbP-Fw   | tatggccgccgATGACATTCCAAAAACGAAAAAGGATG |
| prbP-Rv   | tataggcttcTGCGGCTTTATTTCTTTTCGCTTTT |
| rpoB_Ext-Fw | CGTGTGTTCGCAAGTCTCG                |
| rpoB_Ext-Rv | GGAACCTGGAGCTCATAC              |
| rpoB-Fw   | tatggccgccgCTGGAGATTGACATACCTGATCT |
| rpoB-Rv   | tataggcttcGTAACCTGAAAGGAGAGGATG |

#### OVEREXPRESSION AND PROTEIN PURIFICATION IN p15TV-L

| CLIBASIA_01510_LIC-Fw | TGTATTTCCAGGCG ATGACATTCCAAAAACGAAAAAGGATG |
| CLIBASIA_01510_LIC-Rv | CAAAGCTGGCTCATCA CATATGCCGTCTCTTTGTTTTC |
| CLIBASIA_01510_Ext-Fw | GAGTGTGGAGTGTGTGTGGAAAAAG |
| CLIBASIA_01510_Ext-Rv | CCCACGCAGTTTCTTGAC |

*Restriction sites are underlined.*

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a Spectrolinker XL-1000 UV cross-linker equipped with 312 nm UV bulbs. Biotin labeled DNA was detected using a Phototope-Star Detection Kit (New England Biolabs, Ipswich, MA). Membranes were exposed to Kodak X-ray film.

#### DNase I Footprinting

Protection assays were performed on both minus and plus strands using 5’-6FAM or 5’-VIC labeled probes that were generated by PCR (primers described in Table 2). 1.5 µg of labeled P_pik probe was combined with 40 µM PrbP, 0.5 mM CaCl₂, 2.5 mM MgCl₂, and 0.025 U of DNase I (New England Biolabs) in a 200 µl reaction. After incubation at 37°C for 20 min, the reaction was stopped by the addition of 50 mM EDTA, pH 8.0. As a control, a digestion reaction was performed under the same conditions without PrbP. The digested DNA and the sequencing reaction products were analyzed at the Plant and Microbe Genomics facility, Ohio State University, Columbus, using a 3730 DNA analyzer. The protected regions were identified using GeneMapper software (Life Technology), as previously described (Zianni et al., 2006).

#### Small Molecule Screening by Differential Scanning Fluorimetry

Purified PrbP protein was screened against the Prestwick chemical library of 1152 compounds (Prestwick Chemical, France) using differential scanning fluorimetry as previously described (Vedadi et al., 2006; Niesen et al., 2007; Wrench et al., 2013). Briefly, purified PrbP protein was diluted to a final concentration of 20 µM in 100 mM HEPES, pH 7.5, 150 mM NaCl. 20 µl aliquots of a protein solution containing the chemical compounds were placed in duplicate, into 96-well plates (Bio-Rad) and heated from 25 to 80°C, at the rate of 1°C per min. A real time PCR device (iCycler IQ™, Bio-Rad) was used to monitor protein unfolding by an increase in the fluorescence of the fluorophore SYPRO Orange (Invitrogen). Fluorescence intensities were plotted against temperature for each sample well.
TABLE 3 | Small molecules used in this study.

| Chemical                  | ΔTm | MIC | Concentration | β-galactosidase activity |
|---------------------------|-----|-----|---------------|-------------------------|
|                           | °C  | (µM)| tested (µM)   | (% decrease)            |
| Berberine                 | 1.5 | 100 | 50            | 5.1 ± 0.6               |
| Acetazolamide             | 1.7 | >200| 50            | 3.7 ± 0.4               |
| Metronidazole             | 1.8 | 100 | 50            | 53.7 ± 10               |
| Gramine                   | −2.5| 100 | 40            | 15.3 ± 0.3              |
| Cotine                    | 2.1 | 50  | 10            | 5.2 ± 0.8               |
| Tolfenamic acid           | −4.2| 100 | 25            | 17.5 ± 5.3              |
| Folic acid                | −3.5| >400| 200           | 2.2 ± 0.2               |
| Menadione                 | −2.5| 50  | 15            | 9.8 ± 2.3               |
| 2-Methyl-4(5)-nitroimidazole | ND | >200| 100           | 17.5 ± 2.2              |
| Ornidazole                | 0.5 | 50  | 10            | 11.6 ± 2.4              |
| Dimetridazole             | ND  | 50  | 10            | 25.8 ± 4.6              |
| 1,2-Dimethylidiazole      | ND  | >200| 200           | 37.6 ± 6.1              |
| Ronidazole                | ND  | 10  | 1             | 48.2 ± 3.8              |

The effect of small molecules on the thermal stability of PrbP (ΔTm) and on interactions between PrbP and RpoB (β-galactosidase activity).

ΔTm was calculated as the difference in the transition temperature of PrbP in the absence (PrbP Tm = 37.3°C) and presence of each chemical. The results are the average of duplicates.

MIC: Minimal Inhibitory Concentration for the E. coli TOP10 reporter strain.

Concentration tested: Concentration used in the bacterial two-hybrid system.

β-galactosidase activity (expressed as arbitrary units, AU) as a result of β2H1Δµ-prbP and β2H1Δµ-rpsJ expression is expressed as a decrease in the activity in the presence of chemicals, compared to the control without chemicals after 240 min (OD = 0.8). The assay was performed a minimum of three times, each in triplicates.

and transition curves were fitted with the Boltzmann equation using Origin 8 software (Northampton, MA). The midpoint of each transition was calculated and compared with the midpoint calculated for the reference sample. If the difference between the midpoints was greater than 2.0°C, the corresponding compound was considered to be a “hit.”

Analyses of Chemicals on Infected Leaves

Leaves were collected from HLB-symptomatic Valencia Orange (C. sinensis) trees, maintained at the University of Florida main campus. All leaves used in this study were collected from new flushes on highly symptomatic branches. Prior to treatment all solutions were autoclaved or filter sterilized. 100 µM stocks of the chemical were prepared in 10 mM Tris pH 8.0. A solution of 10 mM Tris pH 8.0 was used for the controls. A scalpel was used to harvest leaves from the tree, with a horizontal cut at the base of the petiole. Each leaf was immediately suspended in 8 ml of treatment solution (with or without chemicals). Leaves were supported in a vertical position throughout the incubation period, with only the lower inch of the petiole submerged in solution (with or without chemical). Steady air flow was maintained over the leaf blades throughout the incubation period, to facilitate transpiration and the adsorption of each solution. Each treatment group consisted of 12 leaves that were processed after 24 h of incubation (with or without chemical).

For each treatment group, biological quadruplicates were prepared from the twelve leaves. The leaf midribs and petioles were collected and incubated overnight at 4°C in RA1aler solution (Life Technologies, Grand Island, NY). Following treatment with RA1aler, samples were rinsed twice with RNase free water and immediately frozen in liquid nitrogen. Samples were then freeze dried over a period of 3 days and homogenized using a GenoGrinder 2000. Homogenized samples were stored at −80°C.

RNA Purification and cDNA Synthesis

Plant and bacterial RNA was extracted from 75 mg of homogenized tissue. Extractions were carried out using the Isolate II RNA Plant Kit (Bioline, London, UK) with lysis buffer RLY. Zirconia beads (0.1 mm) were added to each sample during lysis, to aid with the disruption of bacterial cells. Purified RNA was eluted with 60 µl of RNase/DNase-free water, and subsequently treated with TURBO DNA-free DNase (Thermo Scientific, Wilmington, DE) to eliminate trace amounts of DNA. Purified RNA samples were quantified using a NanoDrop ND 1000 (Thermo Scientific, Wilmington, DE) and stored at −80°C. cDNA was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA) with random hexamer primers and 0.5 µg of RNA. cDNA products were diluted 50% with DNase/RNase-free water and stored at −80°C.

Real-Time Quantitative PCR (qRT-PCR) Analysis

qRT-PCR was carried out in a iCycler IQ apparatus (Bio-Rad) using Platinum SYBR Green qPCR SuperMix for iCycler (Life Technologies) in accordance with the manufacturer’s recommended protocol. Reactions were carried out using 2 µl of cDNA, in a total reaction volume of 26 µl. The genes measured for L. asiaticus included prbP (CLIBASIA_00735), gyrA (CLIBASIA_r05785), rpsJ (CLIBASIA_00325), and 16S rRNA (CLIBASIA_r05785). The mRNA levels of rplK, CLIBASIA_r05785, rpsJ and prbP genes were normalized to the abundance of the plant genes cox2 and 18S rRNA. The expression of each plant control gene was previously examined in absence and presence of tolfenamic acid (data not shown). Quantitative reverse transcription-PCR primers are described in detail in Table 2.

Evaluation of Phytotoxicity on Sweet Orange Seedlings

Twelve-month old seedlings were randomly divided into ten groups of 8 seedlings. Tolfenamic acid was prepared in 10 mM tris buffer, and applied at concentrations of 1, 10, 100, 1000, and 10,000 µM by root soaking (with 100 ml), foliar spray (to saturation), or root soaking and foliar spray combined. A control group was also treated with 10 mM tris buffer only. Each treatment was applied twice at 2 week intervals. A non-treated control group was also maintained throughout the duration of the experiment. Seedlings were monitored for symptoms of phytotoxicity during a period of 12 months following treatment. No phytotoxic effects were observed following treatment with...
tofenamic acid (up to 10 mM). Tolfenamic acid had no effect on the transcription of Cox2 or 18S rRNA (data not shown).

**Evaluation of Antimicrobial Efficacy of Tolfenamic Acid against L. asiaticus**

*Citrus sinensis* “Valencia” inoculation: Twelve-month old seedlings were grafted inoculated with budwood collected from HLB-infected trees. Prior to grafting, the source of the infected tissue was analyzed by PCR to confirm the presence of viable *L. asiaticus*. PCR confirmation was carried out using primers for 16S rRNA (CLIBASIA_r05781) and gyrA (CLIBASIA_00325) genes (see Table 2 for primer sequences). Inoculated plants were kept in a secure greenhouse approved by the USDA Animal and Plant Health Inspection Service (APHIS). The plants were watered in accordance with the standard watering schedule for the commercial citrus industry. Citrus fertilizer (Sunniland Citrus 6-4-6, N-P-K) was applied every 2 months as instructed for the commercial citrus industry. Citrus fertilizer (Sunniland Citrus 6-4-6, N-P-K) was applied every 2 months as instructed for the commercial citrus industry. Citrus fertilizer (Sunniland Citrus 6-4-6, N-P-K) was applied every 2 months as instructed for the commercial citrus industry.

Seedlings treatment study: After 9 months of testing positive for HLB, the highly symptomatic, infected sweet orange seedlings (described above) were randomly divided into two groups. 100 µM tofenamic acid (TA) was applied to one group of inoculated seedlings as a foliar spray (to saturation), and by root soaking (with 100 ml of 100 µM TA). The buffer vehicle (10 mM tris) was applied to the control group as a foliar spray (to saturation), and by root soaking (with 100 ml of 10 mM tris). Each treatment was applied twice, at 2 week intervals. Evaluation of HLB symptoms (leaf yellowing and mottling) and *L. asiaticus* transcriptional activity (as a measure of viability) was performed as described above.

**Statistical Analyses**

qRT-PCR statistical significance was assessed using a two-tail *P*-value, calculated with the Mann–Whitney nonparametric test.

### RESULTS

**PrbP Binds Specifically to the Promoter Region of rplK**

Previous studies in PrbP homologs have found that these proteins may bind non-specifically to the promoter regions of the *rrnA, and rpsH* genes (Gulten and Sacchettini, 2013; Srivastava et al., 2013). In this study, we examined the DNA binding properties of PrbP in *L. asiaticus*, using the promoter region of genes encoding the ribosomal proteins rplK (CLIBASIA_00130; *P*<sub>rplK</sub>) and rpsH (CLIBASIA_00735; *P*<sub>rpsH</sub>), as well as the promoter regions of 16S rRNA (CLIBASIA_r05785; *P*<sub>16S</sub>), *prbP* (CLIBASIA_01510; *P*<sub>prbP</sub>), *dnaK* (CLIBASIA_02620; *P*<sub>dnaK</sub>), and *radA* (CLIBASIA_01095; *P*<sub>radA</sub>). We found that PrbP was able to bind *P*<sub>rplK</sub> at 3.5 µM and *P*<sub>16S</sub> at 5 µM, while binding to *P*<sub>rpsH</sub>, *P*<sub>prbP</sub>, *P*<sub>dnaK</sub>, and *P*<sub>radA</sub> was only observed with higher concentrations (10–15 µM) of PrbP (Figure 1).

### Identification of Small Molecules That Bind PrbP

PrbP was found to compete with the labeled *P*<sub>rplK</sub> fragment, but to a lesser extent than observed with unlabeled *P*<sub>rplK</sub>. The addition of excess (up to 400-fold) unlabeled *P*<sub>radA</sub> or unlabeled *P*<sub>prbP</sub> had no effect (Figure 2). These results indicate that PrbP binds to a specific sequence in the promoter region of rplK, with higher affinity. DNase I footprinting was used to identify the DNA binding site for PrbP in the promoter region of rplK (Figure 3A). The 42 nucleotide protected site (GTAAGTGGTTGTGGTTTTAGAAA), is located 31 bp from the translational start codon, on the plus strand. The critical DNA contact residues were identified by EMSA analysis, following site directed mutagenesis of the rplK promoter region (Figures 3B,C). Of the twelve mutant EMSA probes tested, only rplK-M1, rplK-M4, rplK-M5, rplK-M10, and rplK-M11 resulted in decreased binding to PrbP (Figure 3C). These results indicate that nnnnnnGGTTnnnnnnAAAn is the PrbP recognition sequence for this promoter. These results confirm that *L. asiaticus* PrbP interacts with a specific sequence in the promoter region.

**Liberibacter asiaticus PrbP Interacts with the β-Subunit of the RNA Polymerase**

A bacterial two-hybrid system was used to investigate potential interactions between PrbP and the β-subunit of the RNA polymerase (RNAP). Plasmids pB2HΔα and pB2HΔω were used to create fusions of the *L. asiaticus* genes CLIBASIA_01510 (*prbP*) and CLIBASIA_0011021–150 (*rpoB*, residues 21–150), to the β-galactosidase truncations Δα and Δω as described by Borloo et al. (2007). Protein-protein interactions were followed by β-galactosidase activity. High levels of β-galactosidase activity were observed in strains LBD2 (carrying pB2HΔα-prbP) and pB2HΔω-prpB<sub>21–150</sub> and LBD3 (carrying pB2HΔα-rpoB<sub>21–150</sub> and pB2HΔω-prbP) (5823 ± 345 AU and 3252 ± 217AU, respectively), when compared to the control strains (Figure 4). These results confirm that *L. asiaticus* PrbP interacts with the RNAP.
were found to interact with PrbP to a lesser degree, with a $\Delta T_m$ of 1.5° and 1.7°C, respectively.

**Small Molecules Modulate PrbP Interactions with the RNA Polymerase**

A bacterial two-hybrid system was used to further investigate the interactions between PrbP and RNAP, in the absence and presence of each chemical. The MIC was determined for each chemical in *E. coli* (Table 3). Preliminary $\beta$-galactosidase assays were performed using strains LB01 and LB02, in absence and presence (1 µM–1 mM) of each chemical to determine baseline activity. The highest concentration that did not adversely affect the base levels in the controls was used for subsequent *in vivo* assays (Table 3). Metronidazole showed the strongest effect, decreasing PrbP/RpoB interactions by 53.7%, while berberine, acetazolamide, gramine, cotine, menadione, folic acid, and tolfenamic acid had a lesser effect, decreasing the interaction by 5.1, 3.7, 15.3, 5.2, 9.8, 2.2, and 17.5%, respectively (Table 3).

Consequently, we identified five compounds with chemical scaffolds similar to metronidazole (dimetridazole, 2-methyl-4(5)-nitroimidazole, 1,2-dimethylimidazol, ornidazole, and ronidazole) and their effect was tested on the interaction between PrbP and RpoB. The inhibition values obtained were low for ornidazole (11.6%), 2-methyl-4(5)-nitroimidazole (17.5%), and dimetridazole (25.8%). When tested at higher
concentrations (200 µM), 1,2-dimethylimidazole was found to decrease PrbP/RpoB interactions by 37.6%, however, no effect was observed at lower concentrations (<50 µM). Conversely, a lower concentration (1 µM) of ronidazole was found to significantly decrease (48%) PrbP/RpoB interactions (Table 3).

**Small Molecules Disrupt Interactions between PrbP and DNA**

EMSA were performed with PrbP in the presence and absence of the small molecules identified by differential scanning fluorimetry. The promoter region of rplK (P_{rplK}) was used as the target DNA in all assays. Of the compounds tested, tolfenamic acid was the only effective inhibitor of interactions between PrbP and P_{rplK} (Figure 5A). Complete disruption of the PrbP:P_{rplK} complex was observed with 350 µM tolfenamic acid (Figure 5B). Metronidazole, ronidazole, acetazolamide, gramine, cotine, menadione, and folic acid had no effect on the PrbP:P_{rplK} complex when present at concentrations up to 2 mM.

**Small Molecules as Therapeutics against L. asiaticus**

As propagation of L. asiaticus still remains elusive under laboratory conditions, we used the culturable close relative L. crescens to determine the antimicrobial activity of metronidazole, dimetridazole, ronidazole, and tolfenamic acid. It was found that metronidazole, dimetridazole and ronidazole were not inhibitory at concentrations up to 500 µM. When combined with the results of the bacterial two hybrid system, these results suggest that interactions between PrbP and RNAP may not be critical for the persistence of L. crescens under the conditions tested. Conversely, growth inhibition of L. crescens was observed in presence of tolfenamic acid (70 µM), indicating interactions between PrbP and DNA are indeed essential for the survival of L. crescens (Figure 6).

An infected leaf assay was used to assess the efficacy of tolfenamic acid against L. asiaticus, in vitro (Pagliai et al., 2014). Leaves were collected from Valencia Orange (C. sinensis) trees infected with L. asiaticus. Upon collection, leaves were immediately immersed in 1, 10, or 100 µM tolfenamic acid. Following incubation, each sample was analyzed by qRT-PCR to determine the relative change in gene expression for 16S rRNA, DNA gyrase subunit A, L10 ribosomal, PrbP and RplK protein (encoded by CLIBASIA_r05785, gyrA, rplJ, prbP, and rplK, respectively) as viability parameters for L. asiaticus (Pagliai et al., 2014). Due to the variability in bacterial load, samples were normalized to the plant gene cox2 and 18S rRNA. Samples incubated with 10 or 100 µM tolfenamic acid showed a
significant (p < 0.05) decrease in the expression of all five genes (Figure 7). These results suggest that tolfenamic acid may be inhibitory to *L. asiaticus* at 10 and 100 µM.

The addition of 1 µM tolfenamic acid did not have a significant effect on the expression levels of *gyrA*, *prbP*, or *rplJ*, however, a significant (p < 0.005) decrease in expression was observed for *rplK* and 16S rRNA (93 and 77% reduction, respectively). These results indicate that PrbP is a transcriptional activator for *rplK* and the 16S ribosomal genes in *L. asiaticus*. Additionally, these results indicate that expression of *prbP* is not auto-regulated at the level of transcription, in a manner dependent on DNA binding.

**Use of Tolfenamic Acid as Antimicrobial in HLB-Infected Citrus Seedlings**

Prior to testing the efficacy of tolfenamic acid in *L. asiaticus* infected citrus trees, a phytotoxicity assessment was performed using healthy 12 month-old citrus seedlings. Following 6 months of treatment with tolfenamic acid (1, 10, and 100 µM), no phytotoxic effects were observed in any of the treatment groups. The efficacy of tolfenamic acid was subsequently determined in *L. asiaticus*-infected citrus seedlings (*Citrus sinensis*, "Valencia"). Seedlings were infected with *L. asiaticus* (via grafting) and maintained in a greenhouse for 12 months to allow the infection to spread throughout the entire plant. Each plant had symptoms of advanced *L. asiaticus* infection and the presence of *L. asiaticus* was confirmed by PCR prior to beginning treatments with tolfenamic acid (Figures 8, 9).

Tolfenamic acid (100 µM) was administered to infected seedlings via root soaking and as a foliar spray (to saturation). Two applications were made over a period of 2 weeks. Four infected seedlings were also maintained under the same conditions as a control. Each plant was examined and photographed every 2 weeks to monitor the canopy and root tissue for signs of recovery or disease progression. New root tissue was observed in several of the treated plants 2 weeks after...
β-galactosidase activity was performed using E. coli TOP10 derivatives as reporter strains, transformed with the following plasmid combinations: LB01 (Δ) carrying the empty pB2HΔα and pB2HΔω plasmids; LB02 (♦) carrying pB2HΔα_prbP and pB2HΔω_rpoB21–150; LB03 (□) carrying pB2HΔω_prbP and pB2HΔα_rpoB21–150; and LB04 (○) carrying the empty pB2HΔω and pB2HΔα_rpoB21–150. Assays of β-galactosidase activity are expressed in arbitrary units (AU), and were performed at different growth phases (as indicated by optical density, A600), in triplicates. The β-galactosidase activity for each strain was quantified after subtraction of the baseline activity.

EMSAs were conducted with the biotin labeled probe PrplK (1 ng) and PrbP (2.5 µM), in the absence (lane 2; None) and presence of 2 mM metronidazole, acetazolamide, gramine, cotine, tolfenamic acid, menadione, or folic acid. DMSO (5% final concentration) was ran as a solvent control (lane 3). No protein was added to the first lane. EMSAs were conducted with probe PrplK (1 ng) and increasing concentrations (0–500 µM) of tolfenamic acid, as indicated on top of the panel. PrbP was maintained at 2.5 µM. No protein was added to the first lane.
Two months after treatment, healthy new flush growth began to push on several of the treated plants, and continued to grow over the next 11 months with no visible signs of infection (Figure 8). Eleven months after treatments were applied, three of the four plants treated with tolfenamic acid showed clear signs of recovery in both root and canopy tissues (Figure 8). In contrast, in the control group treated with the buffer vehicle only, three out of the four plants died (Figure 9). In the treated plants, qRT-PCR analysis of root tissue revealed the absence of L. asiaticus infection in plants TA-1, TA-2, and TA-3 while plant TA-4 showed only a 10% reduction in the expression of rplJ (Figure 10). A significant drop (80–95% reduction) in L. asiaticus titer was also observed in the canopy tissue of 75% of the treated plants (Figure 10).

DISCUSSION

Due to the extended timeframe and financial obligations associated with the development and approval of new therapeutic compounds, drug repurposing has become an appealing approach to drug discovery; the availability of pharmacokinetic and pharmacodynamic data can significantly reduce research costs, and expedite the approval process. For these reasons, we elected to use a library of FDA approved compounds as a starting point for screening protein targets. The absence of putative pathogenicity determinants in L. asiaticus, prompted the investigation of unconventional proteins as targets for the development of therapeutic strategies against this devastating pathogen. The in silico analyses revealed a small number of simple regulatory mechanisms in the L. asiaticus genome, which enable its survival during the changing growth conditions encountered in two remarkably different hosts, the psyllid and the citrus plant. As a consequence, the transcriptional regulation of several metabolic pathways must be achieved with only a few transcription factors. Based on these observations, the inactivation of a transcription factor may result in pleiotropic effects, influencing the ability for L. asiaticus to survive within the citrus host.

CarD, a low identity homolog to PrbP, is an essential RNA polymerase binding protein in Mycobacterium species. It is involved in pathogenesis, persistence, cell viability, and resistance to both antibiotics and stress (Stallings et al., 2009; Srivastava et al., 2013). CarD interacts with both the β-subunit of the RNA polymerase and, non-specifically, with DNA sequences in the promoter region of several genes (Srivastava et al., 2013). In L. asiaticus, PrbP also showed interaction with RNAP and DNA. We also found that PrbP was capable of differentially binding the rplK promoter. The putative binding site in the rplK promoter is located 31 bp upstream from the translation start site. This binding location is in agreement with the role of PrbP as a transcriptional activator that may function to stabilize the open promoter complex.

The transcription of rRNA is one of the rate-limiting steps controlling cell growth under ideal and stress conditions; during this process, formation of stable RNAP/open-promotor complexes is the most time consuming step (Zhou and Jin, 1998; Bartlett et al., 2000; Jin et al., 2012; Häkkinen et al., 2013). In Mycobacterium, CarD was recently found to increase the formation of RNAP/open promotor complexes, and stabilize RNAP open complexes by preventing transcription bubble collapse (Davis et al., 2015; Rammohan et al., 2015). Point mutations in CarD that weaken interactions with RNAP are detrimental in M. tuberculosis, resulting in reduced viability and increased sensitivity to antibiotics and oxidative stress (Weiss et al., 2012). As such, we hypothesized that the inactivation/inhibition of PrbP would cause a...
FIGURE 8 | Tolfenamic acid is an effective antimicrobial compound against *L. asiaticus* in HLB-infected citrus seedlings. Recovery of root and canopy tissue in *L. asiaticus*-infected seedling TA-2 (upper panel) after treatment with 100 µM tolfenamic acid. The canopy and root tissue was photographed before treatment with tolfenamic acid, 2 weeks after treatment, 2 months after treatment, and 11 months after treatment. *L. asiaticus*-infected seedlings TA-1, TA-3, and TA-4 are shown in the lower panel. Photographs were taken before treatment began and 11 months after treatment.
decrease in transcript production in *L. asiaticus*, potentially resulting in decreased viability and persistence within the host.

While several compounds were found to modulate interactions between PrbP and RNA polymerase, tolfenamic acid was the only small molecule that decreased interactions between PrbP and DNA (Figure 5). The effect of these chemicals was also tested in vivo, where tolfenamic acid was found to be the only small molecule that affected the overall transcriptional activity of *L. crescens*. The results suggest that the ligand specificity for disruption of the PrbP:PrpK complex is more stringent than the binding specificity for ligands that disrupt interactions between PrbP and RNAP.

To confirm the effect of tolfenamic acid in planta, tolfenamic acid was applied by root soaking and foliar spray to *L. asiaticus* infected citrus seedlings in a greenhouse setting. Each seedling was confirmed to harbor viable, *L. asiaticus* cells for at least 6 months prior to beginning treatment. In addition to testing positive for *L. asiaticus*, each plant also displayed severe signs of infection, including blotchy mottle, yellowing shoots, and severe damage to the root system. After the initial treatment, the first sign of recovery in treated seedlings was the development of new root growth. Rapid recovery of the root system was observed in 75% of seedlings that were treated with tolfenamic acid (Figures 8, 10). Although several plants showed improved root growth in as little as 14 days after treatment, the canopy tissue was slower to recover (5–11 months before substantial amounts of new growth was observed).

The extent of vascular damage caused by the presence of *L. asiaticus* may affect the rate that compounds (chemical treatments) are distributed throughout the canopy, and thus the rate at which each plant recovers. Previous studies have shown that HLB significantly affects the vascular flow and exchange.
of nutrients between root and canopy tissue (Etxeberria et al., 2009; Koh et al., 2012). Factors contributing to the reduction in vascular flow include the accumulation of starch within the xylem and phloem, the blockage of sieve elements by callose formation and bacterial cell debris, and compartmentalization as the tree attempts to isolate the pathogen (Kim et al., 2009; Koh et al., 2012; Aritua et al., 2013). During our analysis of tissue samples collected from HLB-infected seedlings, the highest expression levels of *L. asiaticus* genes (post-treatment) was observed in tissue collected from underdeveloped regions of the canopy, where leaf growth was stagnate. Since the static growth of these branches is indicative of severely obstructed vascular flow, it is possible that the transport of small molecules (from the injection site) to these regions of the tree was insufficient to deliver enough chemical to completely inhibit the growth of *L. asiaticus*. As such, the treatment of highly infected citrus trees may require repeat injections or a combined application method that will facilitate even distribution throughout the canopy tissue (such as a foliar spray with adjuvants). Combined treatments with small molecules and thermotherapy may also be a viable option, as more efficient methods of heat treatment are developed.

There are very few antibiotics approved for use in the treatment of plant diseases, and of those, only streptomycin and tetracycline have shown minimal success in the treatment of HLB, with tetracycline having phytotoxic effects at the concentrations required for treatment of *L. asiaticus*. The combined use of penicillin and streptomycin showed a suppressive effect on *L. asiaticus* (Zhang et al., 2011), however, the use of β-lactam antibiotics is highly regulated and not approved for use in agriculture. As such, the use of small molecules is a promising alternative to combat *L. asiaticus*.

In this study, we were able to confirm that tolfenamic acid is an effective inhibitor of *L. asiaticus in planta*. While additional studies are needed to fully understand the recovery process of treated trees, these encouraging results indicate the potential for tolfenamic acid to be used as a systemic antimicrobial therapy for HLB.

**AUTHOR CONTRIBUTIONS**

CLG, FP, LP, LB, and MT conducted the experiments and analyzed the results. CLG, GL, and CFG conceived the idea for the project, analyzed the results, and wrote the paper.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

A patent application has been submitted for the use of tolfenamic acid for the treatment of HLB.

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