Transcriptome Profiling of ‘*Candidatus Liberibacter asiaticus*’ in Citrus and Psyllids

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ABSTRACT

‘*Candidatus Liberibacter asiaticus*’ (Las) is an emergent bacterial pathogen that is associated with the devastating citrus huanglongbing (HLB). Vectors by the Asian citrus psyllid, Las colonizes the phloem tissue of citrus, causing severe damage to infected trees. So far, cultivating pure Las culture in axenic media has not been successful, and dual-transcriptome analyses aiming to profile gene expression in both Las and its hosts have a low coverage of the Las genome because of the low abundance of bacterial RNA in total RNA extracts from infected tissues. Therefore, a lack of understanding of the Las transcriptome remains a significant knowledge gap. Here, we used a bacterial cell enrichment procedure and confidently determined the expression profiles of approximately 84% of the Las genes. Genes that exhibited high expression in citrus include transporters, ferritin, outer membrane porins, specific pilins, and

genes involved in phage-related functions, cell wall modification, and stress responses. We also found 106 genes to be differentially expressed in citrus versus Asian citrus psyllids. Genes related to transcription or translation and resilience to host defense response were upregulated in citrus, whereas genes involved in energy generation and the flagella system were expressed to higher levels in psyllids. Finally, we determined the relative expression levels of potential Sec-dependent effectors, which are considered as key virulence factors of Las. This work advances our understanding of HLB biology and offers novel insight into the interactions of Las with its plant host and insect vector.

Keywords: Asian citrus psyllid, bacterial pathogens, citrus greening disease, citrus huanglongbing, genomics, NGS, RNA-seq

Citrus huanglongbing (HLB) was first described in China in 1919. Since then, HLB has been reported from all citrus-growing areas worldwide and is considered to be the most devastating disease of citrus (Bové 2006; Wang and Trivedi 2013). Since the introduction of HLB in Florida, in 2005 (Gottwald 2010), citrus production decreased by 70%, causing billions of dollars of economic losses (Hodges and Spreen 2012; Singerman and Rogers 2020). HLB has spread to other citrus-producing states, especially Texas and California, posing an unprecedented threat to the industry (da Graça et al. 2016; Graham et al. 2020).

HLB damages citrus production by decreasing the lifespan of the infected trees, reducing fruit yield, and reducing fruit quality (Bové

2006; Graham et al. 2013). Typical disease symptoms include blotchy mottling and yellowing of leaves; canopy defoliation and dieback; small, deformed, unevenly colored, and off-tasting fruits; premature fruit drop; and loss of fibrous roots and overall root decline, ultimately leading to tree death (Bové 2006; Gabriel et al. 2020). Pathogens associated with HLB are species of the gram-negative bacteria ‘*Candidatus Liberibacter*’. Transmitted by insect vectors, *Liberibacter* colonizes the sieve elements of the phloem tissue in citrus trees. Despite the devastating impact of HLB on the citrus industry, no curative treatment or effective management methods are available to eliminate or mitigate *Liberibacter* in infected trees (Munir et al. 2018), and management of HLB has focused on preventing the spread of the disease by removing infected trees and controlling insect vectors (Grafton-Cardwell et al. 2013). Thus, there is an urgent need to explore new control strategies, which requires a better understanding of the basic biology and virulence mechanisms of *Liberibacter* (Wang 2019; Wang et al. 2017). Unfortunately, this research has been hindered by the inability to grow an axenic culture of pathogenic *Liberibacter* despite enormous efforts. As a result, biological functions and pathways that are important for its colonization in citrus are poorly understood.

Three *Candidatus Liberibacter* species have been found to associate with HLB: *Liberibacter asiaticus* (Las), *Liberibacter africanus*, and *Liberibacter americanus* (Bové 2006). Las and *Liberibacter americanus* are transmitted by the Asian citrus psyllid (ACP; *Diaphorina citri*), and *Liberibacter africanus* is transmitted by the African citrus psyllid (*Trioza erythrae*) (Wang et al. 2017). Las is the most widely distributed species and is associated predominantly with HLB in Asia, Africa, the Middle East, and the Americas (Gottwald 2010; Wang 2019). Because of its low and uneven titer

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in infected citrus, the first genome of Las was obtained from infected insects (Duan et al. 2009). Recently, genome sequences of several isolates from different geographic areas were determined, providing insights into the phylogenetic relationships and genomic variation of Las (Thapa et al. 2020).

Several Las genes have been studied for their potential roles in host interaction and disease development. Flagellin is a well-studied bacterial feature that is often recognized by the plant innate immune system to activate defense response. Although Las harbors genes encoding the flagella system, flagella are formed only in ACP but not in citrus (Andrade et al. 2020). Another gene that may manipulate plant immunity encodes a salicylic acid hydroxylase, which hydrolyzes the plant defense hormone salicylic acid and thus inhibits defense response (Li et al. 2017). Furthermore, a peroxidase encoded by the prophage SC2 may function as a secreted effector and help detoxify reactive oxygen species produced by the host as a defense mechanism (Jain et al. 2015). Finally, proteins secreted through the Sec secretion system have been investigated for their role in facilitating Las colonization in citrus. A core set of Sec-dependent effectors (SDEs) have been identified from Las isolates (Pagliaccia et al. 2017; Thapa et al. 2020), and the virulence activity of some of these SDEs has been characterized (Clark et al. 2018, 2020; Liu et al. 2019; Pang et al. 2020).

Although transcriptome analysis is widely and routinely used to elucidate biological processes, assessment of the Las transcriptome has been challenging because of the extremely small proportion of bacterial transcripts in total RNA extracted from infected tissues. In fact, thorough analysis of bacterial transcriptome in planta has only recently been accomplished for the model pathogen *Pseudomonas syringae* via protocols that either enrich bacterial cells before RNA extraction (Nobori et al. 2018) or enrich bacterial RNAs in total RNA extract (Lovelace et al. 2018). To date, transcriptome studies of HLB have focused on host responses by comparing gene expression changes in healthy and infected citrus or ACP (Kruse et al. 2017; Liu et al. 2020; Wei et al. 2021) and in different citrus varieties with and without infection (Chin et al. 2021; Fang et al. 2021; Hu et al. 2017; Rawat et al. 2017; Wang et al. 2016). However, the Las transcriptomic landscape has not been determined, representing a significant knowledge gap. Here, we evaluated various workflows to optimize RNA sequencing (RNA-seq) outputs for Las by using infected citrus and ACP tissues. Our best-performing protocol allowed the determination of expression levels for 84% Las genes in citrus and 100% in psyllids and facilitated the subsequent analysis of differentially expressed genes in citrus versus psyllids. These analyses provide new information on the cellular processes in Las that might be important for their interactions with the plant host or insect vector.

MATERIALS AND METHODS

Plant and insect materials. Citrus and ACP samples were obtained from fields in Florida and Texas that have been heavily exposed to and affected by HLB. Healthy samples of both hosts were taken as controls. Midrib tissues were collected from two citrus varieties: ‘Rio Red’ grapefruit (*Citrus paradisi* Macfadyenand) and *Citrus macrophylla*. Four biological replicates of HLB-positive ‘Rio Red’ grapefruit were taken from symptomatic, HLB-infected trees that were ≥ 7 years old in a field in Texas. Las-positive *C. macrophylla* and insects (five biological replicates from different colonies) were collected in Florida. Las infection was confirmed, and their titer was determined by reverse transcription quantitative PCR (RT-qPCR) with Las 16S primers (Yan et al. 2013). The cycle threshold (Ct) values of the Las-infected samples were compared with those from the healthy tissues as controls. In all cases, the Ct values from the healthy controls were >32 .

Bacterial isolation from infected citrus tissue. This pre-enrichment procedure was applied to citrus samples via a protocol modified from Nobori et al. (2018). Freeze-dried midribs (from 15 to 25 leaves) were ground to a fine powder in liquid nitrogen. Then

30 ml of fresh bacterial isolation buffer (9.5% ethanol, 0.5% phenol, 2% β -mercapto-ethanol, pH 4.5) was added and mixed thoroughly by vortex. All subsequent processes were done on ice or in a cold room (4°C). The samples were incubated for 20 h with slow shaking and then passed through a Miracloth (EMD Millipore) filter to remove large plant debris. The flowthrough was centrifuged at $3,200 \times g$ for 30 min to pellet plant and bacterial cells. The supernatant was carefully removed, and the pellet was resuspended in 900 μ l of a buffer containing 9.5% EtOH and 0.5% phenol. The suspension was centrifuged at $2,300 \times g$ for 20 min to obtain a two-layered pellet (a white layer on the top and a green layer on the bottom). The top white layer (bacterial cells) was resuspended by pipetting while the bottom layer (plant cells) was left undisturbed. This step was repeated two or three times until a pure white layer was obtained. Bacterial cells were collected by centrifuging at $10,000 \times g$ for 2 min and resuspended in 1 ml of TLE buffer (200 mM Tris-HCl [pH 8.2], 100 mM LiCl, 50 mM EDTA, 10% SDS, 2% β -mercaptoethanol) for RNA extraction.

RNA extraction. All RNAs were extracted from Las-infected samples. For RNA extraction from the citrus midrib, 1 ml of TLE buffer was added to 100 mg of citrus powder. Then 60 μ l of 500 mM CaCl_2 was added to the suspension or the bacterial cells isolated from the citrus tissue as described previously, mixed thoroughly, and placed on ice for 10 min. The solution was centrifuged at $12,000 \times g$ for 10 min at 4°C, followed by two rounds of extraction with 500 μ l of phenol/chloroform/isoamyl alcohol (25:25:1) and centrifugation at $15,000 \times g$ for 10 min at 4°C. The supernatant was mixed with 2 M LiCl and incubated overnight at -20°C. RNA was precipitated by centrifugation at $15,000 \times g$ for 30 min, washed twice with ice-cold 75% ethanol, and resuspended in 20 μ l of ribonuclease (RNase)-free water.

For RNA extraction from ACP, 10 to 15 adult psyllids were ground in liquid nitrogen, immediately suspended in 50 μ l of TRIzol (Invitrogen), and ground until complete homogenization. An additional 950 μ l of TRIzol was added to the homogenate, followed by extraction with 200 μ l of chloroform and final precipitation with 500 μ l of isopropanol. The RNA pellet was washed twice with 75% ethanol and resuspended in 20 to 30 μ l of RNase-free water.

RNA extracts were treated with DNase I (Thermo Fisher Scientific). The RNA concentration and the quality of the extracts were determined with a NanoDrop spectrophotometer and an Agilent 2100 Bioanalyzer with eukaryotic analysis software.

Enrichment of Las RNA. Eukaryotic RNA (18S and 28S rRNAs and polyadenylated RNAs) was removed from total RNA (10 to 25 μ g) extracted from Las-infected tissues with a MICROBEnrich kit (Invitrogen) according to the manufacturer’s instructions. Some of the RNA samples were further treated with a RiboMinus Eukaryote Kit (Invitrogen) to completely deplete 5S and 5.8S rRNA and residual 18S and 28S rRNA. After the MICROBEnrich and RiboMinus Eukaryote Kit treatments, some RNA samples were further treated with poly(A) polymerase (NEB), which adds a poly(A) tail to bacterial messenger RNA (mRNA). The resulting RNA samples were then proceeded for library construction and RNA-seq.

RT-qPCR. We examined the effectiveness of the bacterial RNA enrichment after various treatments by comparing the expression levels of Las 16S rRNA (Yan et al. 2013) and an f-box gene (Citrus Unigene ID CAS-PT-306416) from *Poncirus trifoliata* (Mafra et al. 2012) via RT-qPCR. Primer sequences are listed in Supplementary Table S1. Complementary DNA (cDNA) was synthesized from 1 μ g of total DNase-treated RNA with 300 μ M random primers (Invitrogen) and the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) in a 20- μ l final volume. qPCR was performed in a CFX96 real-time PCR detection system (Bio-Rad) containing 100 ng of cDNA template (2 μ l), 2 $\times$ Quantitect SYBR Green (BioRad), RNase-free water, and 10 μ M gene-specific primers in a final volume of 10 μ l. Cycling conditions were 95°C for 2 min, followed by amplification with 43 cycles of 95°C for 15 s, 54°C for 30 s, and 72°C for 30 s. Relative abundance of the Las RNA was calculated with the

equation $\Delta Ct = Ct_{\text{Las 16S}} - Ct_{\text{citrus Fbox}}$, where the Ct values were the average of two technical replicates.

RT-qPCR for SDE candidates in citrus was performed with the same procedure described previously and primers specified in Supplementary Table S1. The one-step QuantiTect SYBR Green RT-PCR Kit (Qiagen) was used to synthesize the cDNA and perform qPCR under the following conditions: 50°C for 30 min, 95°C for 15 min, and then 40 cycles of 94°C for 10 s, 58°C for 30 s, and 72°C for 30 s. SDE expression was confirmed when Las-positive samples gave a positive Ct value and healthy controls did not allow amplification. For some genes, the primers allowed a minimal amplification with RNA extracted from healthy citrus tissues (confirmed by qPCR with DNA extract and 16S rDNA primers) and gave a high Ct value. In these cases, ΔCt was calculated as $Ct_{\text{Las gene in healthy citrus}} - Ct_{\text{Las gene in diseased citrus}}$, with the average values of technical duplicates and at least two biological replicates. A t test for pairwise comparison (95% confidence) was used to determine whether expression of the SDE was significant.

RNA-seq. Most of the sequencing-ready libraries were constructed with a TruSeq Stranded Total RNA Library Prep Kit (Illumina). The RNA samples were treated with the Illumina Ribo-Zero Plus rRNA Depletion Kit as part of the standard protocol. For the RNA samples treated with poly(A) polymerase, poly(A)-enriched library construction was used. cDNA libraries were then subjected to RNA-seq with an Illumina HiSeq-4000 system with 150-bp strand-specific single-end reads and technical duplicates. Library preparation and sequencing were performed in the DNA Technologies Core Genome Center at University of California, Davis. The total reads per library yielded in these experiments ranged from 83 to 848 million reads.

RNA-seq analysis. The quality of the raw sequencing data was checked with FastQC version 0.11.9 (Andrews 2010). Subsequent statistical analyses were performed in R and Python. To ensure high-quality sequences for mapping and downstream analyses, the reads were trimmed in Trimmomatic version 0.36. The Trimmomatic program comes with standard adapter files (Bolger et al. 2014). RNA-seq reads were aligned with the indexed Las-Psy62 genome assembly (NC_012985.3) and parsed to it in HISAT2 version 2.1.0. The number of reads mapped to each gene was counted via the feature counts function of the Rsubread package (Liao et al. 2019) and are listed in Supplemental Dataset S1. All data used for analysis have been deposited in the GEO database (accession no. 180624).

Differential gene expression analysis. Gene expression values were normalized to counts per million (CPM). Low-expression genes were filtered via the row sum function in R with $\text{cpm} > 0.5$ and $\text{rowSum} \geq 5$ (Robinson et al. 2010). The retained genes included for differential expression had >0.5 CPM in at least four samples (Robinson et al. 2010). Differentially expressed genes (DEGs) were identified from gene counts for each sample with the DESeq2 package version 1.30.1 from Bioconductor (Love et al. 2014). DEGs were selected based on $\log_2$ -transformed and normalized counts that have an adjusted P value below a false discovery rate cutoff of 0.05. Principal component analysis (PCA) of $\log_2$ -transformed read counts was performed for all treatments and replicates with the plotPCA function in DESeq2. The $\log_2$ function transforms the count data to the $\log_2$ scale in a way that minimizes differences between samples with low counts. Hierarchical clustering and Euclidean distance clustering were performed on normalized read counts between treatments and replicates via the dist function and pheatmap function of the pheatmap library version 1.0.12.

Pathway analysis. Gene set (or pathway) analysis was conducted with $\log_2$ fold change ($\log_2FC$) values of normalized mean counts for annotated genes in the Bioconductor package, gauge version 2.24.0. Gene sets of metabolic pathways were obtained from the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database (Kanehisa et al. 2008), with the organismal code “las” used for Las psy62. Significant gene sets were identified from $\log_2$ fold changes between treatments and were selected based on a false discovery rate q -value cutoff of 0.05.

Relevant Gene Ontology (GO) terms were recruited manually from the KEGG gene database, where we determined the IDs of the associated proteins in Uniprot DataBase and extracted their complete GO annotations from QuickGO version 2021-02-08. The GO term analysis was conducted in the biocManager package, ViSEAGO version 1.4.0. The custom GO terms for Las were annotated with Custom2GO and its annotation function. Enriched GO terms were identified for each cluster and DEG set with the entire genome used as the background gene set via the “create_topGO data” function. To test for significant enrichment, the classic algorithm and Fisher’s exact test were used with a cutoff P of 0.05. Redundant enriched GO terms were identified from the REVIGO web server (Supek et al. 2011).

Correlation analysis of the RNA-seq data with published RT-qPCR data. Correlation analysis was conducted on $\log_2FC$ values of Las genes found in citrus midribs relative to those found in ACP samples via the cor.test function in the R stats package version 4.0.4. A total of 225 Las genes with differential relative expression in citrus midribs and ACP were previously evaluated by RT-qPCR by Yan et al. (2013) and Thapa et al. (2020) and were used to compare with our RNA-seq dataset. The entire gene set from each published dataset and gene sets from representative pathways defined by Yan et al. (2013) were evaluated for normal distribution via the shapiro.test function. Correlation was evaluated via Pearson’s correlation test on the normally distributed data from Thapa et al. (2020), whereas Spearman’s test was used on the non-normally distributed data from Yan et al. (2013). For both tests, the significance was evaluated with a P cutoff of 0.05.

RESULTS

Enrichment of Las RNA from infected citrus and ACP tissues. Our understanding of Las biology is hindered by the lack of knowledge of its transcriptome landscape. To overcome this limitation, we first aimed to establish a protocol that enables improved coverage of the Las genome in RNA-seq experiments. Because Las RNA is vastly underrepresented in total RNA extracted from infected host tissues, especially in citrus, several bacterial RNA enrichment methods were explored (Fig. 1).

For citrus tissues, midribs were separated from leaves of grapefruit (*C. paradisi* Macfadyenand) and macrophylla (*C. macrophylla*) trees with HLB symptoms. Las titer in each sample was determined by RT-qPCR with 16S primers (Table 1, Supplementary Table S1). Total RNA was extracted and subsequently subjected to the MICROBEnrich kit (Invitrogen) (one-step treatment) or a combination of MICROBEnrich and the RiboMinus Eukaryote Kit (Invitrogen) (two-step treatment) to deplete plant RNAs, which constitute most of the RNA. The effectiveness of Las RNA enrichment in the resulting RNA samples was evaluated with a bioanalyzer and RT-qPCR in comparison with nontreated samples.

Electropherogram from the bioanalyzer shows a drastic decrease of 28S and 18S rRNA after the one-step treatment (Supplementary Fig. S1). However, the amount of 16S rRNA, represented by absolute intensity, also decreased after the one-step treatment, representing a general loss of RNA or loss of 16S rRNA in addition to eukaryotic rRNA and mRNA. Additional depletion of both prokaryotic and eukaryotic rRNAs was observed after two-step treatment because the rRNA peaks were no longer distinguishable (Supplementary Fig. S1). RT-qPCR results showed a consistent decrease in the relative abundance of Las 16S rRNA over a housekeeping gene of citrus (Table 1) after the successive treatments. This finding indicates that either these treatments were ineffective in enriching Las RNA or 16S rRNA was insufficient to indicate the relative abundance of bacterial RNA after the treatments.

In addition to enriching bacterial RNA from total RNA extract, we also enriched bacterial cells from homogenized midrib tissues via a centrifugation-based procedure (called bacterial isolation herein-after). RT-qPCR results showed that the relative transcript abundances of Las 16S rRNA had consistent increases compared with

the nontreated controls (Table 1), indicating successful enrichment of Las cells and a higher proportion of Las RNA in the RNA extract.

RNA-seq analysis with enriched Las RNA samples. All RNA samples described previously were subjected to RNA-seq. In total, we performed RNA-seq with RNAs from one macrophylla sample, one grapefruit sample, three bacterial cell isolation samples (from grapefruits), and five ACP samples, with technical replicates. Among them, multiple libraries were sequenced for the macrophylla, grapefruit, and two of the ACP samples with RNA subjected to various enrichment treatments (Fig. 1).

Total RNA-seq reads were parsed to the genome assembly available for the psy62 strain of Las. Based on the alignment, the raw read counts corresponding to Las were calculated for each gene, and the read depth represents the total number of reads aligned to the Las genome. A wide range of read depth values and detected Las genes were found among samples and treatments (Table 2, Fig. 2). In general, one-step and two-step enrichment marginally increased the number of reads mapped to Las genes and the coverage of the Las genome; two-step (polyA) treatment actually decreased the number of Las genes that could be detected, although the total reads mapped to the Las genome were drastically increased. This is probably because of the loss of low-abundant Las gene transcripts during the treatment but significant enrichment of Las genes with higher abundance. Compared with the RNA enrichment treatments, RNA extracted from the bacterial cell isolation samples was effective in detecting a higher number of Las transcripts, with ≤ 10 -fold increase in read depth and coverage. For RNA extracted from ACP, the nontreated

samples already had sufficient coverage ($\leq 100\%$) of the Las genome, and the RNA enrichment treatments did not improve the performance.

Euclidean distance analysis and PCA of the $\log_2$ -transformed gene counts for all samples and treatments were performed for

TABLE 1. Comparison of various bacterial cell or RNA enrichment protocols using *Liberibacter asiaticus* (Las)-infected citrus midrib tissues

Sample ^a	Las titer ^b	Treatment ^c	Relative abundance of Las 16S rRNA ^d
Macrophylla	22.82	None	-1.70
		1-step	0.01
		2-step	1.78
Grapefruit 1	23.23	None	-0.94
		1-step	0.16
		2-step	1.87
Grapefruit 2	20.67	None	-5.69
		Bacterial isolation	-6.41
Grapefruit 3	22.44	None	-1.93
		Bacterial isolation	-3.24
Grapefruit 4	22.67	None	-1.49
		Bacterial isolation	-2.28

^a Citrus host and biological replicate for huanglongbing-infected sample.
^b Cycle threshold (Ct) value of Las 16S rRNA determined by reverse transcription quantitative PCR. Healthy tissues were used as controls, which had Ct > 32.
^c No enrichment (none), 1- or 2-step treatments were done with total RNA extract, and bacterial isolation was a treatment before RNA extraction.
^d Relative abundance of Las to citrus genes was calculated as $\Delta Ct = (Ct_{\text{Las 16S}} - Ct_{\text{citrus Fbox}})$. Negative values indicate higher relative abundance of Las.

Fig. 1. RNA sample preparation workflow. Scheme of the experimental procedures examined to increase the proportion of *Liberibacter asiaticus* (Las) RNA in samples prepared from huanglongbing (HLB)-infected citrus and Asian citrus psyllid (ACP) tissues for RNA sequencing (RNA-seq). For RNA enrichment, total RNA extracts were subjected to treatment with MICROBEnrich (one-step) followed by an additional step with a RiboMinus Eukaryote Kit (two-step). Some of the two-step enriched RNA samples were further treated with poly(A) polymerase (polyA) from which bacterial RNAs would be ligated to a poly(A) tail to facilitate poly(A)-enriched RNA-seq library construction. RNA enrichment experiments in citrus were conducted with one biological replicate per variety (macrophylla or grapefruit). The grapefruit midrib tissues were also subjected to a bacterial isolation procedure in order to enrich Las cells before RNA extraction. Bacterial isolation procedures were conducted with three biological replicates. For ACP, four biological replicates were included for the nontreated samples, two for the one-step enrichment samples, and one for the two-step enrichment samples. One or two technical replicates were performed for each biological replicate (Supplemental Dataset S1). All RNA samples were subjected to RNA-seq analyses with nontreated samples as the controls. Except for the polyA treated samples, the RNA samples were further treated using Ribo-Zero Plus rRNA depletion kit as part of the standard RNA-seq library construction protocol. Illustration was made by Biorender (<https://biorender.com>).

quality assessment and to statistically distinguish them in terms of overall gene expression profile (Figs. 2 and 3, Supplementary Dataset S1). The results revealed that the samples grouped together largely based on the host type from which the samples were isolated. Samples collected from ACP and citrus have distinct transcriptome profiles. For example, all macrophylla samples clustered together in the PCA regardless of bacterial isolation or RNA enrichment, indicating that the bacterial or RNA enrichment treatments did not alter Las gene expression profiles. The one-step and two-step treated samples showed similar transcriptome profiles to the untreated controls, further confirming that they were ineffective in enriching Las RNA. A shift in transcriptome profiles in both citrus and ACP was observed after two-step (polyA) treatment, probably because of the substantial loss of low-abundant Las RNA.

Based on the sequencing results and the clustering analyses, we concluded that the RNA extracted from bacterial isolation samples of citrus and the untreated RNA extracted from ACP samples provided the best RNA-seq datasets for each host type. More specifically, these samples gave the most consistent results among biological replicates, demonstrated by the tightest clustering, the best read depth and coverage, and the largest number of Las genes detected. Therefore, these datasets were used for further gene expression analysis.

Las genes with significant expression in citrus. Las has a particularly detrimental impact on citrus compared with ACP. Therefore, we analyzed the Las genes with detectable expression in citrus and focused on the highly expressed genes regardless of their expression in ACP. This analysis indicates which Las functions may facilitate colonization in citrus. To estimate the overall expression of individual Las genes in citrus, we determined the distribution of an average of normalized read counts (CPM) among citrus samples (Fig. 4A). We counted the number of Las genes that fell into 14 groups within the range of CPM from 0 to 15,675. Most genes belong to the first category, with a CPM of 0 to 4, indicating that they have a low expression level. About 140 genes have an average CPM between 4 and 8, and 96 genes have an average of CPM ≥ 8 . This latter group represents the top 10% of the Las genome with the most highly expressed genes in citrus (Supplementary Table S2).

A GO enrichment analysis was performed on these 96 genes, of which 40 were found to be enriched in one or more GO terms. These genes were distributed among several enriched GO terms, as shown in Figure 4B (Supplementary Dataset S3). Many of these genes are linked to housekeeping activities, including primary metabolism, macromolecule biosynthesis, and gene expression. Genes with functions in stress responses, including chaperones and heat shock proteins, and mobile element-related activities, such as transposases, phage capsid, and other phage-related genes, were also enriched. Among other genes worth highlighting are ferritin (CLIBASIA_03035), translocators/transporters, outer membrane porins, Flp family type IVb pilins, and genes involved in cell wall modification. In particular, a potassium transporter (kup, CLIBASIA_03625) had the highest expression among all nonribosomal genes in citrus (Fig. 4A). This gene was also found to be dramatically upregulated in citrus versus in ACP with a $\log_2$ fold change value of 7.45 (Supplementary Table S2). Additional transporters that were found to be highly expressed in citrus include an amino acid ABC transporter (CLIBASIA_00265), an ATP/ADP exchange transporter (CLIBASIA_01040), a LysE family translocator (CLIBASIA_04415), and two Eam A family transporters (CLIBASIA_02310 and CLIBASIA_00915). The EamA family transporters are also highly expressed in citrus compared with their expression levels in ACP with $\log_2$ fold change values of >2 .

As for other genes previously reported to associate with Las infection and HLB development in citrus, CLIBASIA_05590, a prophage-encoded peroxidase (SC2_gp095) (Jain et al. 2015), has an average of CPM of 3.9, and CLIBASIA_00255, encoding the salicylic acid hydroxylase (*sahA*) (Li et al. 2017), has an average of CPM of 1.35. In addition, an SDE candidate, CLIBASIA_03230, showed the highest expression level (CPM of 12.6) compared with other SDEs and is the only SDE that is among the top 10% expressed Las gene in citrus.

Differential expression of Las genes in citrus and psyllids.

We compared the transcriptome profiles of Las in citrus (midrib of grapefruit) and ACP in order to investigate which genes might be important for the HLB disease cycle. Reads per gene were tabulated and statistical analyses were performed. Of the 1,111 annotated genes in the Las genome, 931 and 1,111 had detectable expression in citrus and ACP samples, respectively, corresponding to 84 and 100% coverage. Hierarchical clustering of the complete

TABLE 2. RNA sequencing results from samples with various enrichment treatments

Sample ^a	Treatment ^b	Read depth ^c (×1,000)	Mapped reads (%) ^d	Coverage ^e	Las genes detected (%) ^f
Macrophylla	None	2.3	0.0023	0.28	37.62
	1-step	2.8	0.0030	0.34	35.19
	2-step	1.610	0.0014	0.20	24.66
	2-step polyA	285.1	0.2300	34.84	17.37
Grapefruit 1	None	2.1	0.0026	0.26	47.52
	1-step	2.9	0.0031	0.35	55.81
	2-step	3.6	0.0029	0.44	52.48
	2-step polyA	332.0	0.2700	40.58	31.86
Grapefruit 2	Bacterial isolation	16.1	0.0113	1.97	64.31
Grapefruit 3	Bacterial isolation	17.4	0.0152	2.13	31.46
Grapefruit 4	Bacterial isolation	11.8	0.0110	1.44	43.92
ACP 1	None	41.8	0.0333	5.11	95.77
	1-step	41.5	0.0322	5.07	91.72
ACP 2	None	60.8	0.0465	7.43	96.40
	1-step	32.5	0.0202	3.97	85.60
ACP 3	2-step	1.8	0.0016	0.22	33.48
	2-step polyA	2,735.7	2.2500	334.35	37.89
ACP 4	None	423.3	0.2137	51.73	98.74
ACP 5	None	2,216.4	1.3728	270.88	100.00

^a Citrus host and biological replicate for huanglongbing-infected sample. ACP, Asian citrus psyllid.

^b No enrichment (none), 1- or 2-step treatments were done with total RNA extract, and bacterial isolation was a treatment before RNA extraction. Some RNA samples that had gone through 2-step treatment were further treated with poly(A) polymerase (polyA).

^c Read depth was calculated as total reads mapped to the *Liberibacter asiaticus* (Las) psy62 genome (NC_012985.3) from HISAT2 version 2.1.0 output.

^d Percentage of total sequenced reads that mapped to the Las psy62 genome (NC_012985.3) from HISAT2 version 2.1.0 output.

^e Coverage was calculated as the product of read depth and read length divided by the total genome size, in which the read length is 150 bp and the total genome size for Las psy62 is 1,227,328 bp.

^f Percentage of Las psy62 genes with mapped reads out of the total number of genes in the genome (1,111 genes).

gene set encoded by the Las genome based on their relative expression in each host grouped the genes into 10 clusters, each exhibiting a different expression pattern. After excluding 409 genes, which had low read counts in citrus that are insufficient to support robust statistical analysis, we identified 106 genes with significant differences ($\log_2\text{FCI} > 0$ and adjusted $P < 0.05$) in relative expression between hosts (Fig. 5, Supplementary Dataset S2). Most of these DEGs are distributed across clusters I to VIII, with the majority of the DEGs found in clusters VI and VIII.

Next, the genes in each cluster and the DEGs were subjected to GO analysis (Fig. 6, Supplementary Dataset S3). Significantly

enriched GO terms were identified for each set of genes. In cluster VII, genes related to defense and interspecies interaction were significantly induced in Las during infection of citrus compared with Las found in ACP. These genes include lysozyme-encoding genes CLIBASIA_04790 and CLIBASIA_04800, which could play a role in the restructuring of the bacterial cell wall in response to a defense response in planta. Similarly, in cluster VIII, genes related to transcription, biosynthetic processes, cellular macromolecule metabolism, and translation were also upregulated in citrus.

In analyzing the 106 DEGs for significantly enriched GO terms, we found that genes involved in motility and aerobic respiration

Fig. 2. Summary of RNA sequencing (RNA-seq) results after mapping to the *Liberibacter asiaticus* (Las) psy62 genome. RNA-seq results of gene expression profiles were clustered based on Euclidean distance. The read depth or number of reads mapped to the Las genome and the percentage of total genes with mapped reads are plotted for each sequenced sample. The citrus and Asian citrus psyllid (ACP) samples are designated by a dotted orange and solid blue boxes, respectively. All 22 libraries were included in this analysis (Supplemental Dataset S1). Grapefruit samples that underwent bacterial isolation (three biological replicates and technical duplicates) and ACP samples that received no treatment (four biological replicates) are highlighted with the pink and yellow boxes, respectively, to indicate samples subjected to further analysis.

were repressed in Las isolated from citrus compared with Las colonizing ACP. More specifically, the significantly expressed genes from these GO terms belong to the TCA cycle enzymes such as succinate dehydrogenase and NADH dehydrogenase complexes. Succinate dehydrogenase is a membrane-bound enzyme of the TCA cycle that is involved in the aerobic electron transport pathway to generate energy via oxidative phosphorylation pathways. NADH dehydrogenase is also a membrane-bound enzyme involved in oxidative phosphorylation in the electron transport chain to generate energy in the form of ATP. KEGG pathway analysis supports these findings in that genes involved in the flagella assembly pathway and the oxidative phosphorylation pathways were significantly downregulated in citrus ($q < 0.05$) (Supplemental Dataset S4). For example, the *flaA* gene (CLIBASIA_02090) that encodes flagellin and 10 additional flagella-related genes were highly expressed in ACP. *flaA* has a low expression in citrus (average CPM = 1.83) but showed a $\log_2FC$ of -4.52 in citrus versus ACP, consistent with the previous report that flagella were observed only in ACP but not in plant hosts (Andrade et al. 2020).

Correlation analysis of the RNA-seq results with previously published RT-qPCR data. RT-qPCR analysis has been used to investigate relative expression of individual Las genes in citrus versus ACP. To determine how our transcriptome data align with the

previously published RT-qPCR results, we conducted correlation analyses with $\log_2FC$ values between in planta and in psyllid expression (Fig. 7). In particular, we compared our RNA-seq data with two published studies, which analyzed 198 Las genes (Yan et al. 2013) and 17 SDE genes (Thapa et al. 2020), respectively. Ten of the 198 genes analyzed in Yan et al. (2013) were not included in this analysis because of reannotation of the Las genome. Correlation analysis between the entire gene set from Yan et al. (2013) and our RNA-seq analysis was significant ($P < 0.05$) based on a Spearman’s test, which indicates that there is a relationship between the two datasets; however, with a correlation coefficient value of 0.15, this correlation is weak. The genes analyzed in Yan et al. (2013) were categorized based on specific pathways, so we analyzed the correlation coefficient value for each set of genes separately. Of the seven categories, genes involved in the secretion system had a significant positive correlation with our RNA-seq data ($P < 0.05$, $R = 0.36$). Similarly, correlation analysis with the RT-qPCR results of predicted Las SDEs from Thapa et al. (2020) also revealed a significant positive correlation ($P < 0.05$, $R = 0.52$). The lack of correlation in other gene sets is probably due to the difference in the experimental approach and samples being analyzed.

Expression profiles of predicted SDEs. Because effectors play a key role in disease development, we examined the gene expression

Fig. 3. Principal component analysis of gene expression profile measured in log-transformed bacterial gene counts for huanglongbing-infected hosts. Hosts included in the analysis are grapefruit (orange), macrophylla (green), and Asian citrus psyllid (ACP, blue). Samples were subjected to different treatments: no treatment (none, triangle), bacterial isolation (circle), and RNA enrichment: one-step (crossed square), two-step (cross), and two-step polyA (square). All 22 libraries were included in this analysis (Supplemental Dataset S1). Gene counts from RNA sequencing data were transformed via the *rlog* function in DESeq version 1.30.1.

in ACP, and one (CLIBASIA_00460) was expressed only in citrus (Fig. 8). Of the 15 SDEs expressed in both citrus and ACP, two were identified as significant DEGs: CLIBASIA_05315 (or SDE1) showed significantly higher expression in citrus with $\log_2FC = 4.91$, and CLIBASIA_04410 exhibited significantly higher expression in ACP with $\log_2FC = -1.95$.

When we compared the relative expression of the 24 SDEs in citrus, seven genes were classified as significantly expressed, of which CLIBASIA_03230 had the highest expression in both citrus and ACP (Fig. 8). Because many core SDEs showed low expression, we examined their expression in citrus via RT-qPCR by comparing Ct values between infected and healthy tissues (Supplementary Table S3). Of the eight SDEs that were undetectable in citrus by RNA-seq, five were detectable by RT-qPCR (Supplementary Table S3), indicating that they were probably expressed but at a very low level. Three SDEs were undetectable by both RNA-seq and RT-qPCR, suggesting that they were not expressed, at least in the samples used in this study.

DISCUSSION

This work used a range of new and rigorous methods aimed at establishing the transcriptome landscape of Las in its hosts. We were able to achieve much-improved coverage of the Las genome by RNA-seq with a bacterial isolation protocol modified from Nobori et al. (2018). RNA enrichment treatments aiming to increase

the proportion of Las RNA in total RNA extracts from infected citrus and ACP tissues were also tested. A similar approach was successfully used in other host–microbe systems (Kumar et al. 2016; Lovelace et al. 2018), but it was insufficient to enrich Las RNA. This is probably due to the extremely low abundance of Las RNA in the samples and the inevitable loss of low-abundance RNA during the process. For infected samples with low bacterial titer, as in the case of Las-infected citrus tissues, the best practice would be to use bacterial cell enrichment rather than RNA enrichment for bacterial transcriptome analysis.

Bacterial accumulation in the host types tested in this work was critical to recover sufficient Las genome coverage by RNA-seq. In the case of citrus, we used two species: *C. paradisi* Macfadyen ('Rio Red' grapefruit) and *C. macrophylla*. Samples were collected from naturally infected grapefruits from fields where the disease has effectively established, presumably with Las strain infections (Sétamou et al. 2020). Macrophylla is a commonly used rootstock, where HLB-infected trees showed higher bacterial titers but less severe symptoms (Folimonova et al. 2009; McCollum et al. 2016).

Fig. 5. Differentially expressed *Liberibacter asiaticus* (Las) genes in citrus versus Asian citrus psyllid (ACP). Left; blue/orange heatmap: Hierarchical clustering of the relative expression (RE) of Las psy62 genes in grapefruit (GF) midrib samples (three biological and two technical replicates) after bacterial isolation pre-enrichments and ACP samples with no enrichment (four biological replicates). Middle, green/purple heatmap: Fold changes in Las gene expression in GF samples compared with ACP samples. Right, black/white heatmap: Differentially expressed genes (DEGs), represented by the black bars, based on comparisons between GF and ACP samples where DEGs were identified as genes with $\log_2$ fold change ($\log_2FC$) > 0 and an adjusted $P < 0.05$. Heatmaps were divided into 10 clusters where the total number of genes and total number of DEGs are reported for each cluster.

For both species, we selected highly infected samples with clear HLB symptoms, probably representing a late infection stage.

Based on our RNA-seq analysis, 96 Las genes showed the highest expression levels in citrus (representing the top 10% of the genome),

regardless of their expression levels in psyllids. These genes may reflect the functions important for Las colonization in the plant host. The majority of these genes belong to pathways that are important for basal cellular functions, including gene regulation, transcription,

Fig. 6. Nonredundant significantly enriched Gene Ontology (GO) terms found in *Liberibacter asiaticus* (Las) gene clusters and differentially expressed genes (DEGs). Significant genes for each GO term are plotted based on their log₂ fold changes (log₂FC) in Las gene expression in citrus samples (grapefruit (GF), three biological and two technical replicates) compared with Asian citrus psyllid (ACP) samples (four biological replicates). The gene frequency, represented as the number of significant genes/total number of genes for a given GO term gene group, is indicated by the dotplot color. For a full GO list, see Supplemental Dataset S3. Asterisks indicate log₂FC values significantly different from 0 (two-tailed *t* test at **P* < 0.05, ***P* < 0.01, ****P* < 0.001).

translation, metabolism, and energy production. Recently, Fang et al. (2021) reported comparative Las gene expression in citrus midrib with fruit pith by using dual-transcriptome data. Many genes found to be highly expressed in citrus in their study were also found in this work. For example, ferritin, lysozymes, porins, and chaperones were found in both studies, indicating a role of these genes in the life cycle of Las with possible contributions to

disease development. As one of the most highly expressed Las genes in citrus, the ferritin gene (CLIBASIA_03035) encodes an oxidase that detoxifies excess ferric ions. Iron metabolism and iron-immunity crosstalk were found to be essential in plant immune response (Herlihy et al. 2020; Inoue et al. 2020). It is therefore possible that CLIBASIA_03035 manipulates this process and promotes disease.

Fig. 7. Comparison of RNA sequencing (RNAseq) results with previously published reverse transcription quantitative PCR (RT-qPCR) data. Relationship between $\log_2$ fold change ($\log_2$ FC) values of *Liberibacter asiaticus* (Las) gene expression in citrus versus Asian citrus psyllid (ACP) from our RNA-seq analysis (y-axis) and previously published RT-qPCR analyses (x-axis). Genes were grouped based on categories defined by Yan et al. (2013) including **A**, all 188 genes; **B**, transcription factors (TFs); **C**, transport systems; **D**, motility; **E**, metabolism pathways; **F**, secretion system; **G**, hypothetical proteins; and **H**, other. **I**, Predicted core effectors defined in Thapa et al. (2020). Points represent relative expression of genes, lines represent the regression lines, and shaded areas represent the 95% confidence interval. Spearman's correlation coefficient, R , along with calculated P , is given for all Yan et al. (2013) comparisons. Pearson's correlation coefficient, R , along with calculated P , is given for Thapa et al. (2020) comparison.

Some porins and transporters were highly expressed in citrus. Among them is the outer membrane immunogenic protein OmpL (CLIBASIA_02425). The outer membrane porin OmpA (CLIBASIA_03315) has been used as a detection marker for HLB diagnosis (Ding et al. 2015). With a higher expression level than OmpA, OmpL could be another biomarker to increase HLB detection. CLIBASIA_01685, which encodes a BAX inhibitor-1/YccA protein, is also highly expressed in citrus. The BAX inhibitor is known to have the conserved function as a cell death suppressor in mammals and plants (Hückelhoven 2004). As an obligate pathogen, inhibition of cell death could be critical for the colonization of Las in the hosts. Furthermore, overexpression of YccA in *Escherichia coli* inhibits FtsH-mediated degradation of SecY, a key component of the Sec secretion system (van Stelten et al. 2009). Therefore, it is conceivable that CLIBASIA_01685 may promote Sec secretion and increase the activity of SDE effectors to facilitate infection. Finally, *flp1* and *flp2* (CLIBASIA_03115 and CLIBASIA_03110, respectively), encoding two core Flp (fimbrial low-molecular-weight proteins) family type IVb pilins (Andrade and Wang 2019), are among the top 10% expressed genes in citrus. *flp1* and *flp2* also exhibited significantly higher expression in citrus than in ACP. On the contrary, genes involved in pilus assembly (e.g., CLIBASIA_03080 to CLIBASIA_03040) as well as other pilins, such as CLIBASIA_03105 and CLIBASIA_03095, were upregulated in ACP. Pili have a variety of functions in bacterial attachment, movement, and substrate transport (Hospenthal et al. 2017). It is therefore interesting to hypothesize that Flp1 and Flp2 specifically facilitate the colonization of Las in citrus,

and Las colonization in ACP requires other pilins. Indeed, it was reported that the type IVc tight adherence (Tad) pilus apparatus may contribute to Las colonization of psyllids (Andrade and Wang 2019). It is worth noting that 14 genes out of the 53 significantly upregulated genes in citrus were annotated as encoding hypothetical proteins (as annotated in the KEGG database). Except for CLIBASIA_05315, which has been functionally characterized as a Sec-dependent effector and named *sde1*, the remaining 13 genes have no known functions. They are not predicted to contain N-terminal signal peptide for Sec-dependent secretion (Prasad et al. 2016; Thapa et al. 2020; Yan et al. 2013) and therefore cannot be hypothesized to be effectors of this kind. Nevertheless, their upregulation in citrus might be indicative of important biological functions related to interaction with the citrus host. Future studies to characterize their activities will offer insight on their potential contributions to Las colonization in citrus. Las engages in complex interactions with both citrus and ACP (Galdeano et al. 2020; Huang et al. 2020). In addition to manipulating citrus, Las also causes metabolic alterations in ACP, increasing their mortality rate (Killiny et al. 2017). We found 106 Las genes that are differentially expressed in citrus versus ACP. These genes are distributed throughout the Las genome. Half of them (53) were found to be upregulated in citrus and the other half (53) in ACP. Several transporters were found to be differentially expressed between hosts. For example, three genes encoding metal ABC transporters are significantly upregulated in ACP: CLIBASIA_01135, _02125, and _02120. In contrast, genes encoding a potassium ion transporter

Fig. 8. Expression of predicted core Sec-dependent effectors (SDEs) in citrus and psyllid. The average normalized read counts for each predicted core SDE in citrus (solid bars) and Asian citrus psyllid (ACP; striped bars). Genes are listed in order based on relative in planta expression levels and classified as high (>0 reads, $P < 0.05$), low (>0 reads, $P > 0.05$), or no (0 reads) with RNA sequencing data in citrus, where significance is based on two-tailed t test. Color responds to the correlation with $\log_2$ fold change ($\log_2$ FC) values reported in reverse transcription quantitative PCR (RT-qPCR) studies by Yan et al. (2013) and Thapa et al. (2020).

(CLIBASIA_03625), EamA family transporters (CLIBASIA_02310 and CLIBASIA_00915), an ascorbate transporter (CLIBASIA_05380), and an ATP/ADP exchange translocator (CLIBASIA_01040) were significantly upregulated in citrus samples. Differential expression of different transporters suggests that Las adjusts nutrient uptake during the colonization based on the unique environments of specific hosts including availability of specific nutrients in citrus versus ACP (Li et al. 2012). Genes related to defense and interspecies interactions were also upregulated in citrus. These genes may promote bacterial survival during the infection, by either counteracting plant defense responses or protecting against bacteriophages (Forsberg and Malik 2018). Among this group are genes encoding lysozymes (CLIBASIA_04790 and CLIBASIA_04800) whose immunomodulatory functions in the context of infection might explain their significant upregulation in citrus (Ragland and Criss 2017). Lysozymes are also involved in interspecies interactions and might be relevant to establishing communications or competing with other microbes within the microbial community. These interactions can determine the fitness and evolution of Las in its environment (Blacutt et al. 2020; Gorter et al. 2020; Riera et al. 2017).

Another important group of DEGs was found to be involved in gene expression, biosynthetic processes, cellular macromolecule metabolism, and organic substance biosynthesis such as ribosomal proteins and transfer RNAs. All of them were significantly upregulated in citrus, suggesting that transcription and translation are more active when Las colonizes citrus, consistent with the assumption that Las infection involves genetic reprogramming in planta. However, the activation of these processes in citrus did not correlate with an increase in metabolic activity. On the contrary, it is interesting that genes coding for enzymes participating in energy generation were upregulated in psyllids. This observation can be related to the previous findings, which suggests that Las stimulates ACP to produce more ATP and other energetic nucleotides (Killiny et al. 2017). The TCA cycle was another energy-generating pathway upregulated in ACP. It is interesting to note that Kruse et al. (2017) reported that the expression of ACP genes involved in the TCA cycle was also significantly changed with Las exposure. Another gene cluster that was significantly upregulated in ACP is the flagella system. This is consistent with the previous reports on the downregulation of Las flagellin in citrus (Yan et al. 2013) and the formation of flagella only in psyllids (Andrade et al. 2020).

The RNA-seq data provide novel insight into effector biology in Las. CLIBASIA_05315 or SDE1 was shown to interact with papain-like cysteine proteases in citrus (Clark et al. 2018), promote symptom development (Clark et al. 2020; Pitino et al. 2018), and act as a biomarker for HLB detection (Pagliaccia et al. 2017). Consistent to its important virulence function in citrus, SDE1 is one of the most highly expressed SDEs and almost exclusively expressed in citrus. The SDE candidate CLIBASIA_04410, which encodes a hypothetical protein with predicted domains for a biotin-protein ligase, was expressed in both citrus and ACP but showed significantly higher expression in ACP. Another SDE candidate, CLIBASIA_03230, is equally highly expressed in citrus and ACP. Encoding a hypothetical protein, CLIBASIA_03230 is the highest expressed SDE in both citrus and ACP. How SDEs may manipulate the insect vector is unknown. CLIBASIA_04410 and CLIBASIA_03230 could be good research subjects for studying this overlooked component of HLB biology. It would also be interesting to determine whether CLIBASIA_03230 targets similar cellular processes in plants and insects.

When we compared our RNA-seq dataset with previously published RT-qPCR datasets, only secretion-related genes, especially SDEs, had significant positive correlations with our RNA-seq results. The lack of correlation in other gene sets may be due to differences in experimental designs: Our in planta samples were collected from naturally HLB-infected, symptomatic, adult grapefruit trees found in Texas and macrophylla trees from Florida, whereas the samples from Yan et al. (2013) were collected from symptomatic sweet

orange seedlings that were infected via grafting. Differences in plant age and environmental conditions may also contribute to the low correlation between our RNA-seq data and the RT-qPCR results. Additionally, transcriptomic analyses by RNA-seq allow comparison between different genes under the same condition, whereas PCR-based approaches can only compare the expression level of the same gene under different conditions.

In summary, the current study provides a significant contribution to the knowledge of Las gene expression profiles in naturally infected, mature citrus trees at late infection stages and ACP. The established bacterial cell isolation protocol is a useful tool for similar investigations on the transcriptome of Las and other *Liberibacter* species, as well as other bacterial pathogens with low titers in infected tissue.

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