Transcriptional and Microscopic Analyses of Citrus Stem and Root Responses to Candidatus Liberibacter asiaticus Infection

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Abstract
Huanglongbing (HLB) is the most destructive disease that affects citrus worldwide. The disease has been associated with Candidatus Liberibacter. HLB diseased citrus plants develop a multitude of symptoms including zinc and copper deficiencies, blotchy mottle, corky veins, stunting, and twig dieback. Ca. L. asiaticus infection also seriously affects the roots. Previous study focused on gene expression of leaves and fruit to Ca. L. asiaticus infection. In this study, we compared the gene expression levels of stems and roots of healthy plants with those in Ca. L. asiaticus infected plants using microarrays. Affymetrix microarray analysis showed a total of 988 genes were significantly altered in expression, of which 885 were in the stems, and 111 in the roots. Of these, 551 and 56 were up-regulated, while 334 and 55 were down-regulated in the stem and root samples of HLB diseased trees compared to healthy plants, respectively. Dramatic differences in the transcriptional responses were observed between citrus stems and roots to Ca. L. asiaticus infection, with only 8 genes affected in both the roots and stems. The affected genes are involved in diverse cellular functions, including carbohydrate metabolism, cell wall biogenesis, biotic and abiotic stress responses, signaling and transcriptional factors, transportation, cell organization, protein modification and degradation, development, hormone signaling, metal handling, and redox. Microscopy analysis showed the depletion of starch in the roots of the infected plants but not in healthy plants. Collapse and thickening of cell walls were observed in HLB affected roots, but not as severe as in the stems. This study provides insight into the host response of the stems and roots to Ca. L. asiaticus infection.

Introduction
Huanglongbing (HLB), which is also known as citrus greening, is currently the most destructive disease that affects citrus plants. The disease has been associated with three species of a phloem-limited α-proteobacterium that is designated as Candidatus Liberibacter; i.e., Ca. L. asiaticus, Ca. L. africanus, and Ca. L. americanus [1]. In addition to graft transmission, Ca. L. asiaticus and Ca. L. americanus are transmitted by the Asian citrus psyllid Diaphorina citri, while Ca. L. africanus is transmitted by the African citrus psyllid Triozia citri [1]. While Ca. L. americanus and Ca. L. africanus are still geographically limited, Ca. L. asiaticus is widely distributed, being found in southeast Asia, the Indian subcontinent, the Arabian peninsula, the U.S.A., Cuba, Mexico, Jamaica, Honduras, and Brazil [1].

HLB diseased citrus plants develop a multitude of symptoms, some of which resemble zinc and copper deficiencies [1]. Visible symptoms include blotchy mottled, pale yellow and thin leaves, yellow shoots, corky veins, stunting and twig dieback. Affected fruits are small, lopsided, have aborted seeds, are acidic and bitter in taste, and ripen prematurely [1]. It has also been reported that Ca. L. asiaticus infection seriously affects the roots [2]. It has been observed that Ca. L. asiaticus infected trees are more adversely affected by extreme weather than are healthy trees. Consequently, symptoms of stress, e.g., excessive leaf loss and premature fruit drop, occur in Ca. L. asiaticus infected trees. This stress intolerance is thought to result partially from a loss of fibrous root function. Recently, it was reported that HLB-diseased, four-year-old trees of Valencia sweet orange (Citrus sinensis) on cirtumelo rootstock (Citrus paradisi x Poncirus trifoliata) showed a 31% and 38% reduction in fibrous root mass density for presymptomatic and symptomatic trees, respectively, compared to healthy trees. Similarly, HLB-diseased, three-year-old trees of Hamlin sweet orange (C. sinensis) on cirtumelo rootstock showed a 30% and 37% reduction in fibrous root mass density for presymptomatic and symptomatic trees, respectively [3].

Anatomical aberrations in the Ca. L. asiaticus infected leaves compared to healthy leaves include the excessive accumulation of starch, callose depositions, plasmolysis plugging, necrosis and collapse, the swelling of sieve elements and companion cell walls, and the disruption of chloroplast inner grana structures [1,4–8]. Analyses of secondary metabolites in the Ca. L. asiaticus infected fruit compared to healthy fruit revealed increases in terpenes,
hesperidin, naringenin, quercetin, limonin and nomilin agycones [9,10]. Starch accumulates excessively only in aerial tissues, such as the phloem elements and vascular parenchyma in leaves and petioles, xylem parenchyma and spongy mesophyll cells and the phellem of stems, but it is depleted in roots [4,6,8]. While sucrose and fructose accumulate in both the leaf midribs and lobes, glucose accumulates only in the midribs [7]. In addition, the disruption of inner grana structures occurs only in leaf parts that are experiencing phloem plugging [5]. Rosales and Burns [11] also showed that within the same fruit, the indole-3-acetic acid concentration is higher in misshapen areas compared with those that are normal in shape. Previous studies indicate that Ca. L. asiaticus is distributed in bark tissue, leaf midrib, roots, and different floral and fruit parts, but not in endosperm and embryo, of infected citrus trees [12]. The leaves, stems, and roots play distinct roles in the photosynthesis and transportation of water, nutrients, etc. However, the effects of Ca. L. asiaticus infection on gene expression in the stems and roots remain to be elucidated despite the recent progress that has been made toward understanding the transcriptomes of leaves and fruit that are infected with Ca. L. asiaticus using either microarray or RNA-seq [4,13–18].

Microarray analyses of infected sweet orange (Citrus sinensis) leaves revealed that Ca. L. asiaticus modulates a large cascade of molecular pathways [4,13,14,17], to which some of the above-mentioned phenotypes have been attributed. For example, in addition to callose deposition, the plugging of phloem elements has been linked to phloem protein 2 (PP2), which contributes to starch accumulation [4,5,13]. It was suggested that the excessive starch accumulation is partly due to the up-regulation of starch synthesis genes, such as ADP-glucose pyrophosphorylase (AGPase), starch synthase, granule-bound starch synthase (GBSS) and the starch debranching enzyme (SDE) [4,13]. Recently, Fan et al. [7] reported the down-regulation of starch metabolism enzymes, such as transglucosidase and a maltose exporter. Furthermore, their work demonstrated an increase in cell wall invertase activity in HLB-affected citrus plants, which may lead to increases in sucrose and glucose. Not surprisingly, several biological processes differentially modulated in leaves during the symptomatic phase of Ca. L. americanus infection were similarly affected by Ca. L. asiaticus infection including induction of transcripts encoding zinc transporters [14], induction of transcripts encoding key enzymes involved in starch biosynthesis and repression of those related to starch breakdown, and induction of transcripts encoding P-proteins and repression of transcript encoding a salicylic acid-binding protein 3 (SABP3) [19].

Both microarray and RNA-seq techniques have been used to investigate the citrus fruit response to Ca. L. asiaticus infection [15,18]. Liao and Burns [18] compared the transcriptomic changes associated with Ca. L. asiaticus infection in flavedo, vascular tissue, and juice vesicles from symptomatic, asymptomatic and healthy fruit based on microarray analysis. Their results indicated that many categories of metabolism including numerous genes involved in carbohydrate transport and metabolism were affected by HLB, but no category appeared to be specific to the disease. It was also suggested that mechanisms regulating development of HLB symptoms may result from the host disease response rather than being a direct consequence of carbohydrate starvation [18]. RNA-Seq was used to profile the transcriptome of citrus fruit response to Ca. L. asiaticus infection focusing on the peel [15]. Importantly, RNA-Seq can reveal rare and unknown transcripts and expression of genes unrepresented on the arrays, and avoid non-specific hybridization despite some drawbacks of RNA-Seq [15,20–22]. It was shown that numerous pathways including those involved in photosynthesis, source-sink communication, sucrose and starch metabolism, and hormone synthesis and signaling were differentially regulated by Ca. L. asiaticus infection [15]. Proteomics analyses also helped us understand the host response of citrus to Ca. L. asiaticus infection [14,23]. In this study, we compared the gene expression levels of stems, and roots in healthy Valencia sweet orange trees with those that were infected with Ca. L. asiaticus using microarrays. Microscopy analyses were also conducted to compare the stems and roots of healthy and HLB diseased citrus.

Results

To understand how Ca. L. asiaticus infection affects stems and roots, Affymetrix microarray analysis was conducted. After removal of probe set-related redundancies, and at a P value of <0.05 and a log2 fold change (LFC) of ≥1.00 or ≤−1.00 as cutoff thresholds, a total of 988 genes showed significantly altered expression, of which 885 were in the stems, and 111 in the roots. Of these, 551 and 56 were up-regulated, while 334 and 55 were down-regulated in the stem and root samples, respectively (Fig. 1). Dramatic differences in the transcriptional responses were observed between the citrus stems and roots to Ca. L. asiaticus infection, with only 8 genes affected in both the roots and stems. For the identification of the processes and genes that were affected, the data was analyzed using the MapMan gene ontology system [24]. We found that the affected genes are involved in diverse cellular functions (Fig. 2, Fig. S1, Fig. S2), including carbohydrate metabolism (Fig. S3), cell wall biogenesis (Fig. 2), biotic and abiotic stress responses (Fig. 3), signaling and transcriptional factors (Fig. 3, Fig. 4, Fig. S2, Fig. S4), secondary metabolism (Fig. S5), phenylpropanoid pathway (Fig. S6), transportation, cell organization, protein modification and degradation, development, hormone signaling, metal handling, redox, and enzymatic activities (Fig. S1, Fig. S2). The proportions and types of genes that belonged to the various functional groups were different between roots and stems, with the most categories altered in the stems compared with the roots. The low similarities between the stem and root expression profiles indicate that Ca. L. asiaticus infection affects the stems, and roots differently. Based on previous knowledge regarding the responses of citrus plants to Ca. L. asiaticus infection, we focused further analyses on seven gene categories that are presumed to significantly contribute to the HLB disease symptoms caused by Ca. L. asiaticus infection.
Starch and Sugar Metabolism

The induction of starch accumulation by Ca. L. asiaticus infection has been found to be in the leaves followed by stems but not in the roots [4,6,7]. Sucrose and glucose accumulation by Ca. L. asiaticus infection has also been found in the leaves [4,7]. Therefore, we compared the expression of carbohydrate metabolism genes in the stems and the roots. In conformity to the above, the expression of the genes encoding enzymes and proteins that are involved in both major and minor carbohydrate metabolism was only affected in the stems, but not in the roots (Fig. 2, Fig. S3, Table 1). The transcription of genes encoding an ADP-glucose pyrophosphorylase large subunit 3 (APL3), which catalyzes the rate limiting step in starch synthesis [25], and a granule-bound starch synthase (GBSS), which initiates synthesis of starch granules, was up-regulated. Among the genes encoding starch-degrading and sucrose cleaving enzymes, the genes for alpha-amylase 1 (AMY1) and exocellular acid invertase 1 (Exinv1) were up-regulated, while those encoding beta-amylase 1 (BMY1) and a neutral invertase were down-regulated. Ca. L. asiaticus infection also repressed the transcription of minor carbohydrate metabolism associated genes encoding trehalose biosynthesis enzymes, trehalose-6-phosphate synthases (TPS2, TPS8, and TPS10) and two trehalose-6-phosphate phosphatases, but activated the gene for myo-inositol oxygenase (EC 1.13.99.1), which cleaves inositol to D-glucuronic acid (Fig. 2, Table 1).

Cell Wall Metabolism

Plant cell walls are composed of layers of cellulose microfibers embedded in a matrix of pectin and hemicellulose, plus some structural proteins [26–28]. Ca. L. asiaticus infection induced differential expression of multiple genes encoding enzymes and proteins involved in the synthesis, assembly, and modification of cell wall. The infection repressed the expression of genes encoding cellulose synthase-like D4 (CSLD4) and CSLC7 that mediate β-1,4 linkages in the hemicellulose backbones, and a cellulose synthase catalytic subunit protein (Fig. 2, Table 1).
Disease Resistance and Pathogenesis-related (PR) Genes

The expression of disease resistance associated genes belonging to the nucleotide binding site (NBS)-leucine-rich repeat (LRR) gene family that contain centrally located NBS, C-terminal LRR and amino-terminal TIR (Toll/interleukin-1 receptor-like) or CC (coiled-coil) domains were affected predominately in the stems compared to the roots by Ca. L. asiaticus infection (Table 2). In the stems, reduced transcription was observed for genes encoding a NBS-LRR-like protein C17, a TMV N-like disease resistance protein that confers resistance to tobacco mosaic virus in tobacco [37], a putative TIR-NBS-LRR-class protein, and an inter-alpha-trypsin inhibitor heavy chain-related protein (Table 2). The genes encoding a CC-NB-LRR domain containing protein, a resistance protein candidate 2 (RGC2), and a disease resistance family protein SC0A belonging to the Cladosporium fulvum resistance protein Cf-9 (Hcr9) family, were however, up-regulated (Table 2).

In the roots, while the expression of a gene encoding the CC-NB-LRR was repressed, expression of a gene encoding a NBS-LRR protein Hom-F was up-regulated by Ca. L. asiaticus infection. Also specifically in the stem, genes encoding miraculin-like proteins, which are soybean Kunitz-type trypsin inhibitors that specifically inhibit the activities of membrane-bound serine proteases [38], were up-regulated. Among PR proteins, only the transcription of a PR10-encoding gene was up-regulated in the stems but was unaffected in the roots (Table 2).

Signaling Molecules and Receptor-like Kinases

The expression of several genes encoding receptor-like kinases (RLK) which regulate plant development and defense response was affected in the stems but not in the roots (Fig. 3; Table 3). Ca. L. asiaticus infection up-regulated expression of 12 plant defense genes (Fig. 3) such as homologs of Xa21, Hr2-5D, Cf-5 and Cf-2.2, and a gene encoding a systemin receptor SR160 which was reported to induce systemic defense genes in wounded tomato plants [39]. Five development related genes including genes encoding a receptor protein kinase protein ERECTA and a receptor-like protein kinase 1 were down-regulated in the stems by Ca. L. asiaticus infection (Table 3). Ca. L. asiaticus infection up-regulated genes encoding FERONIA (FER) in the catharanthus roseus-like gene family [26] and WAK-like kinase (WLK2) in the wall-associated kinase gene family (Table 3). Cell wall-associated kinase (WAK) and WLKs family gene products which differ from RLKs by containing a cytoplasmic serine/threonine kinase domain and an extracellular region containing epidermal growth factor-like repeats regulate cell elongation and plant development as well as pathogen and heavy-metal tolerance [40].

The expression of genes that are involved in transducing Ca²⁺ signals was altered by Ca. L. asiaticus infection. In the stems, expression of eight genes was repressed including genes encoding a calcium-dependent protein kinase Atr9/Cf-9 rapidly elicited protein (ACRE), a calcium-binding allergen Ole e 8, a calmodulin-binding protein, an EF-hand Ca2+-binding protein CCD1, and a 39 kDa EF-hand-containing protein but only one gene encoding calreticulin was up-regulated (Table 3). In the roots, only the transcription of a gene for calcium binding pollen allergen, polcalcin, was activated (Table 3). Among genes whose products are involved in sugar sensing and nutrient physiology, genes encoding a glutamate receptor (GLR) family protein GLR3.3, a phosphate-responsive protein, and a phosphate-induced 1 (PHI-1)-like protein were up-regulated in the stems (Table 3). The gene encoding a photoassimilate-responsive protein PAR-1a was of a gene encoding a polygalacturonase-like protein which hydrolyses galacturonic acid residues in cell walls (Fig. 2, Table 1).
repressed in stems, but up-regulated in the roots (Table 3). *Ca. L.* asiaticus infection also up-regulated the expression of a gene encoding an ionotropic glutamate receptor homolog GLR4 (Table 3).

**Transcription Factors**

*Ca. L.* asiaticus infection up-regulated the expression of a gene encoding an Arabidopsis response regulator 1 (ARR1) homolog but repressed the expression of a gene encoding a lateral organ boundaries domain protein 38 (LOB38) in the roots (Fig. 4, Fig. S4, Table 4). LOBs perform numerous developmental functions and are known to be expressed in boundaries of the shoot and at the base of secondary roots [41,42]. In the stems, expression of four genes encoding AUX/IAA family proteins was repressed by *Ca. L.* asiaticus infection (Table 4). Meantime, expression of an auxin response factor (ARF10) gene was also repressed (Table 4). Genes encoding basic helix-loop-helix (bHLH) family proteins showed both patterns of regulation (4 repressed and 2 up-
regulated). Noticeably, the transcription of a gene encoding a homeobox-leucine zipper protein was repressed in the stems but was up-regulated in the root (Table 4). Ca. L. asiaticus infection repressed the expression of three genes but induced one gene belonging to the APETALA2/Ethylene-responsive element binding family in the stems and in the roots respectively (Table 4). Expression of two MYB domain TF family genes including a tuber-specific and sucrose-responsive element binding factor gene were up-regulated in the stems, whereas in the roots three genes in the same family including MYB68 were up-regulated (Table 4). Importantly, its homolog AtMYB68 is a root growth specific regulator, impacting overall plant development under unfavorable conditions [45]. MYB68 is a negative regulator of lignin deposition in Arabidopsis [43], and its down-regulation may affect lignification in Ca. L. asiaticus infected citrus. Ca. L. asiaticus infection only affected the expression of genes encoding proteins containing WRKY domain in the stems, wherein, it increased the expression of WRKY4 and WRKY23 but repressed that of WRKY30 (Table 4). Overexpression of WRKY4 enhanced susceptibility of Arabidopsis plants to P. syringae and suppressed PR1 gene expression [44]. The effect of up-regulation of WRKY4 in Ca. L. asiaticus infected citrus remains to be addressed. Zinc finger-containing transcriptional factors that are involved in plant developmental and defense showed both patterns of regulation in the stems (Table 4). Expression of the following genes were repressed in the stem: ZPT2-12, a member of the EPF-type zinc fingers that are involved in flower development and plant defense against pathogens [45]; a gene encoding a CCCH-type zinc finger family protein; and a gene of the C2C2/Zn-GATA family that has been implicated in light-responsive gene transcription in Arabidopsis [46], and a gene of the C2C2/Zn-CO-like family that regulates circadian clock-responsive genes [47]. Ca. L. asiaticus infection induced the expression of a gene of C2C2/Zn-DOF family that are involved in the regulation of carbohydrate metabolism and plant defense [48,49] in the stems and in the roots, respectively. With the exception of a gene encoding an AP2 SCARECROW (SCR)-like (SCL) protein, expression of three genes belonging to the GRAS transcription factor family was repressed in the stems, including SCL14 and a scarecrow gene regulator highly related to chitin-inducible gibberellin-responsive proteins (Table 4). SCL and SCL proteins are involved in GA-mediated regulation of plant growth [50]. Arabidopsis mutants of SCR showed aberrant root growth and impaired development in aerial plant organs such as hypocotyl, indolorecence stems and shoot axial organs [51,52]. Some of these symptoms are similar to those induced by Ca. L. asiaticus.

### Transportation

The expression of numerous genes that are involved in transportation of carbohydrates, drugs, water, oligosaccharides, and amino acids was altered by Ca. L. asiaticus infection in the stems (Table 5). Among the carbohydrate transporters, Ca. L. asiaticus infection repressed the expression of a sugar transporter gene CiSUT1 but increased the transcription of genes encoding a hexose transporter 6 (HEX6) and a sorbitol transporter in the stems. In the roots, only the transcription of a sorbitol transporter gene was repressed (Table 5). Expression of three genes encoding amino acid transporters was up-regulated whereas one was down-regulated in the stems. Only one gene whose expression was up-regulated, encoding a putative amino acid transporter highly

### Table 2. Differentially expressed genes related to disease resistance and pathogenesis-related proteins in the stems and roots of Valencia sweet orange (Citrus sinensis) caused by Ca. L. asiaticus infection.

| Accession No. | Gene description | Log, fold change |
|--------------|------------------|-----------------|
|              |                  | Stem | Root |
| CK670605     | PR protein from class 10 | 1.03 |       |
| CK674271     | Pathogenesis-related protein PR10A | 1.43 |       |
| DN618117     | NBS-LRR-like protein cd7 | −1.32 |       |
| CK674648     | TMV N-like disease resistance protein | −1.64 |       |
| CK671207     | Inter-alpha-trypsin inhibitor heavy chain-related | −1.04 |       |
| CB322091     | Disease resistance protein (TIR-NBS-LRR class) | −1.67 |       |
| CD573651     | NBS-LRR type disease resistance protein Hom-F | 1.05 |       |
| CK075252     | CC-NB-LRR protein | −1.06 |       |
| DN796858     | CC-NB-LRR protein | 1.21 |       |
| DN798196     | Resistance protein RGC2 | 1.28 |       |
| CK641603     | Disease resistance protein, putative | 1.05 |       |
| CK287601     | Disease resistance family protein, SCDA | 1.99 |       |
| CK934670     | Miracinulin-like protein 2 | 2.72 |       |
| CK305265     | Miracinulin-like protein 2 | 3.38 |       |
| CK043805     | Miracinulin-like protein 2 | 3.72 |       |
| CK674833     | Miracinulin-like protein 2 | 5.37 |       |
| CK045518     | Miracinulin-like protein 2 | 3.82 |       |
| CF838881     | Miracinulin-like protein 3 | 3.39 |       |

Accession No. is a unique identifier of EST sequences from several citrus species and hybrids linked to the NCBI. LFC is the ratio of the expression level in the infected samples compared to the healthy trees. The ratio is the mean of 3 replicates. The annotation is according to the latest available BLASTx search at non-redundant protein database at the NCBI. Metabolic pathway grouping is based on the gene ontology in the MapMan program (Thimm et al., 2004). doi:10.1371/journal.pone.0073742.t002
Table 3. Differentially expressed genes related to signaling in the stems and roots of Valencia sweet orange (*Citrus sinensis*) caused by *Ca. L. asiaticus* infection.

| Accession No. | Gene description | Log2 fold change |
|---------------|------------------|-----------------|
|               |                  | Stem | Root |
| Crinkly like  |                  |      |      |
| CV711750      | putative Protein kinase | -1.06 |     |
| Lysine motif  |                  |      |      |
| CX293076      | putative Protein kinase | -1.01 |     |
| Wall associated kinase |                  |      |      |
| CD574549      | WAK-like kinase (WLK2) | 4.88  |     |
| Catharanthusroseus-like RLK |                |      |      |
| DN622543      | FERONIA receptor-like kinase | 1.15  |     |
| MAP kinase    |                  |      |      |
| CX073386      | MPK3             | -1.44 |     |
| Receptor like cytoplasmatic kinase |                |      |      |
| CN184216      | protein Serine threonine kinase-like | -1.25 |     |
| CV707629      | Receptor protein kinase-like protein | -1.14 |     |
| CX670980      | putative Protein kinase | 1.58  |     |
| CV719828      | AT3G57730        | 1.16  |     |
| DN617645      | Receptor protein kinase | 2.43  |     |
| DUF 26        |                  |      |      |
| CN185603      | hypothetical protein F19K19.4 | -1.39 |     |
| CN192263      | AT1G01540        | -1.13 |     |
| DN625179      | Receptor protein kinase-like protein | 1.70  |     |
| CV70863       | putative Receptor-like protein kinase 4 RLK4 | 1.65  |     |
| CV706414      | Receptor protein kinase | 1.24  |     |
| CX674957      | hypothetical protein F20P5.3 | 1.93  |     |
| CX640171      | putative Serine threonine kinase | 1.60  |     |
| CX295060      | putative Serine threonine kinase | 1.07  |     |
| DN617645      | Receptor protein kinase | 2.43  |     |
| Leucine rich repeat |             |      |      |
| CV707041      | probable Receptor kinase | -1.28 |     |
| CX675277      | putative Receptor protein kinase, ERECTA | -1.57 |     |
| CX043848      | putative Receptor protein kinase, ERECTA | -1.37 |     |
| CX671538      | Hcr2-SD         | 2.37  |     |
| CK935615      | Calcium-binding allergen Ole e 8 | -1.59 |     |
| CK935615      | Calcium-binding allergen Ole e 8 | -1.59 |     |
| DT214563      | putative Systemin receptor SR160 | 2.51  |     |
| DN623196      | Receptor protein kinase-like protein | 2.02  |     |
| CK669844      | Receptor-like protein kinase 1 | -1.18 |     |
| Calcium signaling |               |      |      |
| DN622169      | Avr9/Cf-9 rapidly elicited protein | -1.16 |     |
| CK935615      | Calcium-binding allergen Ole e 8 | -1.59 |     |
| CN188862      | Calcium-binding EF-hand family protein-like | -1.91 |     |
| CK701540      | Calcium-binding pollen allergen, Polcalcin | 1.13  |     |
Table 3. Cont.

| Accession No. | Gene description | Log2 fold change |
|---------------|-----------------|-----------------|
| C047037       | EF-hand Ca²⁺-binding protein CCD1 | −1.49 |
| CX06727       | 39 kDa EF-Hand containing protein | −1.37 |
| DN15254       | putative Calmodulin-binding protein | −1.63 |
| DN93677       | putative Calmodulin-related protein | −1.62 |
| DN799085      | putative Calreticulin | 1.43 |
| DN33994       | unnamed protein product | −1.07 |

Sugar and nutrient physiology

| Accession No. | Gene description | Log2 fold change |
|---------------|-----------------|-----------------|
| CX94586       | Glutamate receptor family protein (GLR3.3) | 2.63 |
| CF837692      | Ligand gated channel-like protein | 1.10 |
| CV711517      | phosphate-induced protein 1(PH1)-like protein | 1.60 |
| CF838115      | Phosphate-responsive protein, putative | 1.15 |
| CX92843       | Photoassimilate-responsive protein precursor PAR-1a | −1.70 2.12 |
| CV710376      | Ionotropic glutamate receptor homolog GLR4 | 1.03 |
| CX88476       | putative Ligand-gated ion channel subunit | 1.50 |

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related (e-value, 4e-41) to the amino acid/polyamine transporter II of Medicago truncatula, was identified in the roots. Ca. L. asiaticus is auxotrophic for at least five amino acids [53]. It remains to be determined whether the up-regulation of such transporter genes results from the metabolic incapacity on certain amino acids of Ca. L. asiaticus.

Among metal transporter genes, Ca. L. asiaticus infection activated the expression of genes encoding two zinc transporters (such as ZIP1), but repressed that of a metal tolerance protein B1 (MTPB1) (Table 5). In addition, expression of genes encoding transporters that move peptides, oligopeptides, and ions across membranes, including a POT family gene, NRT1 and YSL5, was up-regulated in the stems but not in the roots (Table 5). Expression of genes whose products perform various functions such as PUP1 (purine permease 1) which is involved in the uptake and transport of cytokinins [54]; urca transporter DUR3 involved in acquisition, transportation, and utilization of urca [55]; and a chloride channel-like (CLC) protein which moves chloride ions across membranes, and two multidrug and toxic compound extrusion (MATE) efflux family proteins was also up-regulated (Table 5). The expression of a gene encoding a nucleobase ascorbate transporter 12 (NAT12) was up-regulated in the roots (Table 5).

Secondary Metabolic Pathway

The expression of genes encoding proteins and enzymes that synthesize flavonoids, isoprenoids, phenylpropanoids and lignin was mostly up-regulated by Ca. L. asiaticus infection (Table 6). Only the expression of one gene encoding a naringenin-chalcone synthase 4 (CHS4) was repressed in the stems. Ca. L. asiaticus infection induced the transcription of 10 genes in the isoprenoid metabolic pathway such as genes encoding a 1-deoxyxylulose 5-phosphate synthase which catalyses the rate-limiting step in the production of isopentenyl diphosphate, the main precursor of all isoprenoids, a beta-amyrin synthase, a geranyl diphosphate synthase small subunit, a homogentisate geranylgeranyl transferase, two linalool synthases, and a gamma-terpinene synthase (Table 6). Expression of genes involved in the phenylpropanoid pathway were similarly up-regulated including genes encoding phenylalanine ammonia lyase (PAL; EC 4.3.1.5), a key enzyme that converts P-phenylalanine to trans-cinnamic acid, a precursor for various phenylpropanoids [56], a hydroxycinnamoyl transferase (HCT; EC 2.3.1.135), which catalyzes the conversion of p-coumaroyl CoA or caffeoyl CoA with shikimic acid to p-coumaroyl shikimate or caffeoyl shikimate; as well as a 10-hydroxygeraniol oxidooreductase, a caffeic acid O-methyltransferase II, the 4-coumarate-CoA ligase-like protein and catechol O-methyltransferase (Fig. S6, Table 6). The expression of only one gene encoding a transferase family protein closely related to anthranilate N-benzoyltransferase involved in phenylpropanoids pathway was induced in the roots (Table 6).

Validation of Differentially Expressed Genes by Quantitative Reverse Transcription PCR (qRT-PCR)

To confirm the validity of the microarray experiment, qRT-PCR assays were performed. Seven genes encoding the SBIP1A, PRP4, CAT5, PAR-1a, GLR4, the tuber-specific and sucrose-responsive element binding factor (TSF), and ZIP1 were chosen for this confirmation (Table 6). These genes were chosen because they showed distinct patterns of expression. Common patterns of expression changes were observed by the two methods, indicating the reliability of the microarray data (Table 7).

Microscopy Analyses of Stems and Roots Infected by Ca. L. asiaticus

Light microscopy analyses of the stems of healthy and Ca. L. asiaticus infected plants were shown in Fig. 5. Phloem fibers were observed on the outer boundary of the phloem layer, with cambium and xylem on the inner boundary (Fig. 5). The difference between the stems from the healthy and Ca. L. asiaticus infected plants observed with light microscope was the increased amount of cell layers in the phloem and the increased thickness of the cell walls of
Table 4. Differentially expressed genes related to transcriptional factors in the stems and roots of Valencia sweet orange (Citrus sinensis) caused by Ca. L. asiaticus infection.

| Accession No. | Gene description | Log2 fold change |
|---------------|------------------|------------------|
|               |                  | Stem | Root |
| AP2/EREBP, APETALA2/Ethylene-responsive element binding protein family | | |
| CF836437      | putative AP2 domain transcription factor | 1.27 | |
| CN185220      | Transcription factor TINY, putative | −2.56 | |
| CF831712      | AP2 domain transcription factor-like protein | −1.80 | |
| CK939963      | CaCBF1B | −2.12 | |
| ARF, Auxin Response Factor family | | |
| CK294853      | Auxin response factor 10 | −1.17 | |
| Arabidopsis response regulator (ARR) | | |
| BQ623949      | Response regulator1 (ARR1) | 1.19 | |
| AS2, Lateral Organ Boundaries Gene Family | | |
| CF834266      | LOB domain protein 38 | −1.59 | |
| Aux/IAA family | | |
| CD574798      | AUX/IAA protein | −2.21 | |
| DR404247      | AUX IAA protein | −1.18 | |
| CV704184      | AUX IAA protein | −1.07 | |
| CV708756      | Auxin-regulated protein | −1.46 | |
| bHLH, Basic Helix-Loop-Helix family | | |
| CX045057      | Basic helix-loop-helix (bHLH) family protein | 2.63 | |
| CK932760      | bHLH transcription factor, putative | −1.04 | |
| CX676159      | Helix-loop-helix DNA-binding protein-like | −1.60 | |
| CF506528      | putative bHLH transcription factor | −1.60 | |
| CX671833      | putative Transcription factor RAU1 | 1.39 | |
| CX044856      | unknown protein | −1.11 | |
| bZIP transcription factor family | | |
| CF507428      | bZIP transcription factor | −3.04 | |
| C2C2(Zn) CO-like, Constans-like zinc finger family | | |
| CX048448      | Zinc finger (B-box type) family protein | −1.62 | |
| C2C2(Zn) DOF zinc finger family | | |
| DN798578      | unknown protein | 1.49 | |
| CX640084      | unknown protein | 1.10 | |
| C2C2(Zn) GATA transcription factor family | | |
| CX049639      | putative protein | −1.08 | |
| Zinc finger family | | |
| DN622949      | Zinc finger protein, ZPT2-12 | −1.46 | |
| DN797852      | Zinc finger protein, ZPT2-12 | −1.52 | |
| CK939958      | Zinc finger (CCCH-type) family protein | −1.56 | |
| GRAS transcription factor family | | |
| CN186577      | Scarecrow gene regulator | −1.62 | |
| C8291917      | Scarecrow transcription factor family protein | −1.25 | |
| CX670106      | Scarecrow-like transcription factor 14 (SCL14) | −1.14 | |
| CV710984      | AP2 SCARECROW-like protein | 1.01 | |
| HB, Homeobox transcription factor family | | |
| DN798602      | Homeobox leucine zipper protein | −1.42 | 1.21 |
| CV704865      | Homeobox 2 protein | 1.06 | |
| MYB domain transcription factor family | | |
| CX307046      | MYB-like DNA-binding domain protein | 1.68 | |
| CK936024      | MYB family transcription factor (MYB68) | 1.30 | |
| CF834992      | MYB-related transcription factor | −1.42 | 1.20 |
some of the cells in this area (arrows). When observed at higher magnification (TEM), the following were observed for the stems of *Ca. L. asiaticus* infected plants: a swelling in the middle lamella (Fig. 3 D, E) in some areas and, in others, the increase in thickness and collapse of cell walls (Fig. 5 E, F) that appeared to be those of the sieve elements (SE) and companion cells (CC). This collapse observed in the stem was similar to, but not as severe as, that reported in petioles and mid-ribs of leaves affected by HLB. There was also typically more starch (S) in the HLB affected tissues (Fig. 5 C, D, Fig. 6 E, F) compared to the healthy.

The comparison of healthy and HLB affected roots did not reveal as marked a contrast as in the stem (Fig. 7). The major differences included more starch (S) found in the roots of healthy plants (Fig. 7 A, B) compared to the HLB affected root (Fig. 8 C, D). There was not an increase in the development of phloem cells in the HLB roots as there was in the stem tissue (Fig. 6). At the light microscope level, the collapse and thickening of cell walls of HLB affected roots (Fig. 7 C, D arrows) were not as severe as in the stems (Fig. 5). At the higher magnification (TEM), thickening of the middle lamella (ML) (Fig. 4, E) and thickening and collapse of the sieve elements (CSE) (Fig. 4 F) were also observed in the HLB affected roots. Another difference observed was the apparent greater density of the cytoplasm (Fig. 8 D) in the HLB affected phloem compared to that in the cells of the healthy plant (Fig. 8 A, C). Nuclei (N) and other organelles (Fig. 8 E, F) in the phloem parenchyma (PP) and companion cells (CC) in HLB affected roots were more prominent than in the healthy roots.

### Discussion

Dramatic differences in the transcriptional responses were observed between the citrus stems and roots to *Ca. L. asiaticus* infection (Fig. 1). Overall, 885, and 111 genes were regulated in the stems, and roots, respectively, by the infection, with only 8 genes overlapping (Fig. 1). In contrast to the low similarity between the expression profiles of the stems and roots, the expression profile of the stem to *Ca. L. asiaticus* infection is very similar to the leaf [4, 13–15, 17, 57]. For example, when we compared our data with the leaf expression profile [4], 291 genes were regulated in both the stems and the leaves compared to the 8 genes shared between the stems and roots (Fig. 1). The low similarity among the stem and root expression profiles might result from that *Ca. L. asiaticus* affects the stems and roots differently due to the distinct functions of the stems and roots, and the different physiological changes caused by the pathogen. Depletion of starch in the roots while excessive starch accumulation in the stems have been reported in *Ca. L. asiaticus* infected plants previously [4, 6, 8] as well as in this study (Fig. 5, Fig. 7). We could not rule out other possibilities that might contribute to the expression differences between the roots and the stems such as the stems and the roots are in different stages of infection since the plants were graft-transmitted. The collapse and thickening of cell walls in HLB affected roots (Fig. 7 C, D) were not as severe as in the stems (Fig. 5) suggesting that roots were in earlier stage of infection compared to the stems. The early stage of infection of the roots by *Ca. L. asiaticus* probably contributes to that only 111 genes were significantly affected in expression in roots.

The overall expression pattern of the stems to *Ca. L. asiaticus* infection shares high similarity with that of the leaves. High similarity between the expression profiles of the stems and the leaves might be due to that they share some common functions and have some similar phenotypes to *Ca. L. asiaticus* infection. The accumulation of starch, sucrose and glucose as induced by *Ca. L. asiaticus* has been found to be highly concentrated in both the leaves and stems (Fig. 5, Fig. 7) [4, 6, 7]. Collapse of sieve elements of the stems (Fig. 5) was similar to, but not as severe as the leaves (data not shown). The expression pattern of the stems to *Ca. L. asiaticus* infection suggests a very similar virulence mechanism of the pathogen as we learned from the leaf expression pattern including affecting carbohydrate metabolism, phloem blockage and aberrations, cell wall, plant defense, and hormones [58]. The excessive accumulation of starch in the stems (Fig. 7) is due to the similar mechanism as that of the leaves. This accumulation has been attributed to callose deposition, sieve pore plugging by the PP2 protein, and phloem necrosis and collapse [4, 13–15, 17, 57]. In addition to the above, other factors, including limited starch breakdown due to the down-regulation of starch-degrading genes, such as amylases and disproportionating enzyme 2 (DPE2), also contribute to the starch accumulation. Starch is degraded byAMY to maltose, and by BMY (BAM) together with DPEs to maltose and glucose. *Ca. L. asiaticus* infection increased the expression of AMY1 but repressed that of BMY1 (Table 1). While AMYs play minor roles, BMYs are indispensable for the breakdown because they catalyze the rate-limiting step [59]. Fan et al. [7] linked the accumulation of soluble sugars, such as glucose and fructose, to the up-regulation of cell wall-bound invertases. In our study, expression of the gene encoding Exinv1 was also up-regulated in the stems (Table 1).

Both the expression patterns of the stems and leaves suggest that *Ca. L. asiaticus* infection resembles a susceptible interaction between citrus and the pathogen [4, 13–15, 17, 57]. *Ca. L. asiaticus* infection repressed the expression of multiple genes that contain

| Accession No. | Gene description | Log2 fold change |
|---------------|------------------|-----------------|
| CF506570      | Tuber-specific and sucrose-responsive element binding factor | -2.69 |
| CK939682      | putative WRKY transcription factor 30 (WRKY30) | -1.91 |
| AJ489051      | putative WRKY4 transcription factor | 3.16 |
| CK058028      | putative WRKY-type DNA binding protein 23 (WRKY23) | 1.51 |
| CK643787      | WRKY transcription factor | 1.32 |

Accession No. is a unique identifier of EST sequences from several citrus species and hybrids linked to the NCBI. LFC is the ratio of the expression level in the infected samples compared to the healthy trees. The ratio is the mean of 3 replicates. The annotation is according to the latest available BLASTx search at non-redundant protein database at the NCBI. Metabolic pathway grouping is based on the gene ontology in the MapMan program (Thimm et al., 2004).

**Table 4. Cont.**

| Accession No. | Gene description | Log2 fold change |
|---------------|------------------|-----------------|
| CX050828      | putative WRKY transcription factor | 1.51 |
| CX643787      | WRKY transcription factor | 1.32 |
Table 5. Differentially expressed genes related to transportation in the stems and roots of Valencia sweet orange (*Citrus sinensis*) caused by Ca. L. asiaticus infection.

| Accession No. | Gene description | Log2 fold change |
|---------------|-----------------|-----------------|
| **Sugar transporter** | | |
| CK936504 | putative Sorbitol transporter | −1.41 |
| DN958552 | Sorbitol transporter | 1.25 |
| CB292174 | Hexose transporter, putative | 1.28 |
| CK665499 | Hexose transport protein HEX6 | 1.28 |
| CK936487 | Citrus sucrose transporter 1 (CiSUT1) | −1.16 |
| **Amino acid transporters** | | |
| CF418599 | Amino acid transporter family protein | 1.26 |
| CV713475 | putative Cationic amino acid transporter | −1.02 |
| CK98643 | Amino acid transporter family protein | 1.16 |
| CF832082 | Cationic amino acid transporter 5 (CAT5) | 1.62 |
| CK935365 | putative Amino acid transport protein | 1.45 |
| **Metabolite transporters** | | |
| CK939426 | Mitochondrial substrate carrier family protein | −2.05 |
| **Major Intrinsic Proteins (NIP, PIP, SIP)** | | |
| CK638658 | Major intrinsic protein | −2.47 |
| DN958192 | NOD26-like intrinsic protein 6;1 | 2.71 |
| CK644460 | putative Aquaporin (PIP1-1) | −1.50 |
| CV719411 | Plasma membrane aquaporin (PIP1A) | −1.03 |
| AU300362 | Putative aquaporin (PIP1-3) | −1.38 |
| CK933592 | Small basic membrane integral protein 1A (SIP1A) | 1.68 |
| **Metal transport** | | |
| CK297247 | Metal tolerance protein B1 (MTPB1) | −1.02 |
| DN919440 | putative Zinc transporter | 1.48 |
| CB290596 | Zinc transporter protein, ZIP1 | 3.76 |
| **Peptides and nucleotide transport** | | |
| CN189143 | putative Proton-dependent oligopeptide transport (POT) family | 1.51 |
| BQ622927 | Yellow stripe like 5 (YSL5) | 1.51 |
| DN922714 | Nitrate transporter 1 | 1.57 |
| CX305691 | Nitrate transporter NRT1-2 | 3.72 |
| **Phosphate and potassium transport** | | |
| CX044970 | Phosphate transporter 3 | 1.29 |
| CX047721 | Potassium channel tetramerisation domain-containing protein | −1.08 |
| **Vesicle transport and secretory pathways** | | |
| CK293833 | putative Coatamer protein complex, subunit beta 2 | 1.74 |
| CV708721 | unknown protein | −1.66 |
| CK295788 | CAO | −1.18 |
| CV888526 | VAMP family protein | −1.03 |
| CF653150 | VAMP family protein | −1.09 |
| CF837201 | putative RNA-binding protein | −1.49 |
| **ABC transporters and multidrug resistance systems** | | |
| CB292087 | putative ABC transporter family protein | 3.11 | −1.80 |
| CK668058 | P-glycoprotein-like protein | 1.28 |
| CK93439 | putative MRP-like ABC transporter | 1.11 |
| **Miscellaneous** | | |
| CK671045 | putative protein | −1.04 |
| CV709319 | Purine permease 1 (PUP1) | 1.37 |
| CV707033 | Urea transporter DUR3 | 1.61 |
NBS-LRR domains (Table 2). NBS-LRR proteins belong to a major class of disease resistance molecules that recognize pathogenic effectors or plant proteins that are targeted by effectors, and many map to the R (resistance) gene loci [60]. They activate defense signaling [60]. The down-regulated genes encode homologs of the NBS-LRR-like protein cD7, the Tobacco mosaic virus (TMV) N-like disease resistance protein [37], and a putative CC-NBS-LRR gene that is related (e-value of 2e-43) to the potato (Solanum tuberosum) CC-NB-LRR class gene that was rapidly activated by an incompatible race of Phytophthora infestans [61]. The down-regulation of these genes indicates that Ca. L. asiaticus infection represses the defense response of citrus, leading to the susceptibility. Interestingly, our previous study indicated that Ca. L. asiaticus infection encodes a functional salicylate hydroxylase (SahA) that converts SA into catechol, which does not induce resistance [58]. SA has been proven to be an endogenous resistance signal, and its derivative MeSA is one of the signals for systemic acquired resistance (SAR). The accumulation of SA and its derivatives are necessary for SAR because it has been shown that plants that are unable to accumulate SA through the transgenic expression of a bacterial salicylate hydroxylase (NahG) that metabolizes SA into catechol are deficient in SAR. The expression of the bacterial salicylate hydroxylase enzyme in planta led to decreased SA levels, the failed development of SAR, or the decreased expression of the PR genes and heightened susceptibilities to both virulent and avirulent pathogens [62,63]. On the other hand, Ca. L. asiaticus infection also activated the transcription of numerous defense related genes including genes encoding PR10, CC-NB-LRR protein, and disease resistance family protein SC0A (Table 2). However, the induction of plant defense related genes is incapable of preventing the establishment of Ca. L. asiaticus in the phloem. Collectively, the expression pattern of plant defense related genes to Ca. L. asiaticus infection resemble that of the susceptible plant pathogen interactions [64,65].

We observed certain expression pattern associated with the stems that was not identified previously with the leaves. For example, Ca. L. asiaticus infection repressed expression of genes encoding the pore-forming aquaporins PIP1A, PIP1-1, PIP1-3 while induced expression of SIP1A gene, that belong to the MIP gene family, in the stems (Table 5), where the long distance movement of substances is common. Aquaporins are important molecules in plant physiology because they facilitate the transportation of water and other small, uncharged solutes, such as glycerol, CO2, ammonia and urea, from sources, such as roots and leaves, through the stem to other plant parts [66–68]. Their repression in citrus by Ca. L. asiaticus infection can deprive affected plants of water and some essential nutrients. Additionally, Ca. L. asiaticus infection caused dramatic effect on the phloem (Fig. 5, Fig. 6) in the stems as the major transportation pathway, which might contribute to the disease symptoms in the roots (Fig. 7, Fig. 8). On the other hand, the detrimental effect of HLB on roots will have negative feedback effect on the aerial parts of the plant.

Interestingly, swelling in the middle lamella was observed in both Ca. L. asiaticus infected stems and roots, which has not been reported previously (Fig. 6, Fig. 8). Swelling in the middle lamella was also observed in the presymptomatic leaf of Ca. L. asiaticus infected citrus [8]. The adjacent cells were separate from each other as observed in Fig. 6 and Fig. 8 which might affect nutrient transport among the neighboring cells via plasmodesmata, leading to eventual cell death. However, Ca. L. asiaticus does not encode plant cell-wall degradation enzymes such as cellulases, pectinases, xylanases, or endoglucanases [53]. Ca. L. asiaticus might indirectly affect the middle lamella by interfering with the plant cell wall related enzymes encoded by citrus such as rhamnogalacturonate lyase which are known to be regulated by plant hormones such as abscisic acid (ABA) and auxins [69]. Ca. L. asiaticus was reported to affect plant hormones including IAA and ABA in the fruit [11]. A detailed analysis of hormones in different tissues at different infection stages is required to test this hypothesis in the future. It remains to be determined how Ca. L. asiaticus causes the anatomical changes in middle lamella and how those changes affect HLB symptom development.

In conclusion, we analyzed the host response of citrus stems and roots to Ca. L. asiaticus infection. Dramatic differences in the transcriptional responses were observed between the citrus stems and roots to Ca. L. asiaticus infection. Microscopy analyses indicated that Ca. L. asiaticus infection significantly affects the starch accumulation, and sieve elements of the stems or roots. Understanding how to reduce the adverse effect of Ca. L. asiaticus infection on the leaves, stems and roots is critical to manage HLB.

Materials and Methods

Source of Plant Materials

Two-year-old Valencia sweet orange (C. sinensis) on rootstock Swingle citrumelo (Citrus paradisi, Macf. × Poncirus trifoliata [L.] Raf.) plants used in this study were graft-inoculated with budwood from Ca. L. asiaticus infected citrus trees and maintained in a USDA-APHIS/CDC-approved secured greenhouse. The inoculated plants that were used in this experiment were Ca. L. asiaticus-free before the graft inoculations, as shown by PCR and Q-PCR tests using specific primers [12]. Stem and root samples were obtained from three HLB symptomatic trees and three healthy control trees of similar size and from similar positions approximately 16 months after inoculation. The presence of Ca. L.
asiaticus in the plants was confirmed using both conventional and quantitative PCR as described previously [12].

Microarray Analysis

Total RNA from the stems and roots were isolated from freshly obtained samples using the RNeasy Plant Mini Kit and treated with DNase (Qiagen, Valencia, CA). Root samples were prepared by excising small pieces from lateral roots and were frozen in liquid nitrogen before RNA purification. Stem pieces were harvested from young stems bearing symptomatic leaves by peeling off the bark and phloem together. For the softer stem parts, the whole stem was cut into smaller pieces and processed, as described above for the roots. The samples were grinded in liquid nitrogen with a mortar and pestle, the powder was rapidly suspended in RLT buffer (Qiagen, Valencia, CA) that was supplemented with 1% mercaptoethanol, and the solution was processed using the RNeasy Plant Mini Kit according to the manufacturer’s instructions. The quality of RNA was checked using the NanoDrop™ 1000 spectrophotometer (NanoDrop Technologies, Inc.), and only samples with A260/280 and A260/230 nm ratios of ~2.0 were selected. The integrity of the

| Accession No. | Gene description | Log2 fold change |
|---------------|------------------|------------------|
|               |                  | Stem  | Root |
| Flavonoids    |                  |       |      |
| CX672036      | putative Flavanone 3-hydroxylase | 2.21  |      |
| CX045954      | Naringenin-chalcone synthase 4 | ~1.14 |      |
| Isoprenoids   |                  |       |      |
| CX302245      | 1-deoxyxylulose 5-phosphate synthase | 1.15  |      |
| CF838068      | Beta-amaeryn synthase | 1.58  |      |
| CX290062      | Geranyl diphosphate synthase small subunit | 2.70  |      |
| CX667086      | GGPP synthase     | 1.28  |      |
| CX943706      | HMG-CoA synthase 2 | 1.22  |      |
| CX665915      | Homogentisate geranylgeranyl transferase | 2.14  |      |
| CV886253      | Linalool synthase | 2.51  |      |
| CV885575      | Linalool synthase | 3.41  |      |
| CX045048      | Gamma-terpinene synthase | 3.75  |      |
| CX671596      | Acetyltranferase-like protein | 1.29  |      |
| CK937255      | Acetyltranferase-like protein | ~1.23 |      |
| Phenylpropanoids/lignin biosynthesis | | | |
| CX671596      | Acetyltranferase-like protein | 1.29  |      |
| CX044256      | Hydroxycinnamomyl transferase | 2.12  |      |
| CF830793      | Transferase family protein | 1.47  |      |
| CX663894      | Transferase family protein | ~1.11 |      |
| CV884611      | 4-coumarate-CoA ligase-like protein | 1.33  |      |
| CV705977      | 4-coumarate-CoA ligase-like protein | 1.14  |      |
| CB291954      | 10-hydroxygeraniol oxidoeductase | 2.08  |      |
| DN622570      | putative Orcinol O-methyltransferase | 3.86  |      |
| CX043719      | Caffeic acid O-methyltransferase II | 1.73  |      |
| CX303448      | Catechol O-methyltransferase | 1.34  |      |
| CX670983      | Catechol O-methyltransferase | 2.53  |      |
| CX302017      | O-methyltransferase | 1.34  |      |
| CX643181      | Phenylalanine ammonia-lyase 2 (PAL2) | 3.62  |      |
| CF835217      | Phenylalanine-ammonia lyase | 2.55  |      |
| Amino acid synthesis | | | |
| DN620167      | Prephenate dehydrogenase family protein | 1.24  |      |
| CF834506      | Prephenate dehydratase family protein | 1.10  |      |
| CV706063      | 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase | 1.75  |      |

Accession No. is a unique identifier of EST sequences from several citrus species and hybrids linked to the NCBI. LFC is the ratio of the expression level in the infected samples compared to the healthy trees. The ratio is the mean of 3 replicates. The annotation is according to the latest available BLASTx search at non-redundant protein database at the NCBI. Metabolic pathway grouping is based on the gene ontology in the MapMan program (Thimm et al., 2004).

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total RNA was further determined using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA).

Microarray experiments were carried out at the Gene Expression Core Facility of the Interdisciplinary Center for Biotechnology Research at the University of Florida. Data analyses were conducted as described previously [4]. Briefly, the Affymetrix GeneChip was used for microarray analysis. The GeneChip Citrus Genome Array contains 30,171 probe sets representing up to 33,879 citrus transcripts based on EST sequences obtained from several citrus species and citrus hybrids. The raw data were normalized using the robust multichip analysis (RMA) approach [70]. Linear models were used to assess the differential expression, while the empirical Bayes method was used to moderate the standard errors [71]. Differentially expressed genes were ranked by P values and fold changes. Genes with a cutoff threshold P value of 0.05 and LFC of $\geq 1.00$ or $\leq -1.00$ were considered to be differentially expressed. To generate diagrams of the metabolic pathways and biological processes that

| Table 7. Comparison of expression of selected genes in the stems and roots of Valencia sweet orange (Citrus sinensis) to Ca. L. asiaticus infection based on quantitative reverse transcription-PCR and microarray analyses. |
|---------------------------------------------------------------|
| **Gene product description** | **Microarray** | **qRT-PCR** |
|                              | **Stems** | **Roots** | **Stems** | **Roots** |
| CK933592 Small basic membrane integral protein | 1.68 | 3.65 |
| CF417841 Proline-rich protein PRP4 | 1.09 | 1.40 |
| CF832082 Cationic amino acid transporter 5 | 1.62 | 2.13 |
| CX292843 Photoassimilate-responsive protein PAR-1a | 2.20 | 4.96 |
| CV710376 Ionotropic glutamate receptor homolog GLR4 | 1.03 | 2.42 |
| CF506570 Tuber-specific and sucrose-responsive element binding factor | $-2.69$ | $-2.93$ |
| CB290596 Zinc transporter protein ZIP1 | 3.75 | 2.56 |

*Fold change value calculated according to the method of Livak and Schmittgen (2001). The following primers designed using the PrimerQuest™ program (Integrated DNA Technologies, Inc) were used, CK933592: TGCAGTGTTGACATCTCTGTTGGGT/ACTGGTAACAGGGCTTCAACTCCA; CF417841: ATCAGGCACTCCATGTCAGCGTTA/GTATAAGCAGCGGTTGAAGCAGCA; CF832082: TGCAGCCTCTCTTGTGAGGAGATA/TCCCAAGAACAAAGAAAGGAGGAG; CX292843: TATGCGAAGCACAAGGGAAAGGTG/AAAGTTCCGGTTATGTGGCACCGGC; CV710376: TGCAGCCTCTCTTGTGAGGAGATA/TCCCAAGAACAAAGAAAGGAGGAG; CB290596: AAGGGATATTCAACGCAGCAGCAG/ACAAGGACATGCAACCAGCTCCTA.

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diagrammatic comparison of the metabolic pathways and biological processes that contribute to the development of HLB. The results of this study provide new insights into the molecular mechanisms underlying HLB and may facilitate the development of strategies to control this devastating disease.
families whose expression were significantly altered. Details of our microarray experiments and the MIAME-compliant microarray data have been deposited in the Gene Expression Omnibus database, the National Center of Biotechnology Information (Accession Number GSE33004).

Quantitative Reverse Transcription PCR (qRT-PCR) Assays

Expression of seven genes was confirmed using qRT-PCR and the same set of RNA that was used for the microarray. Primers were designed using the PrimerQuest™ program (Integrated DNA Technologies, Inc.). All of the qRT-PCR reactions were performed using total volumes of 25 μl in an ABI PRISM 7900 sequence detection system (Applied Biosystems, Foster City, CA) with the QuantiTect SYBR Green RT-PCR Kit (Qiagen, Valencia, CA) and ∼50 ng of total RNA. 10 nM PCR primers, 0.25 μl RT mix (Qiagen, Valencia, CA) and 12.5 μl QuantiTect SYBR Green RT-PCR Master Mix (Qiagen, Valencia, CA). The PCR conditions were 30 min of reverse transcription at 50°C followed by 15 min of predenaturation at 95°C and 40 cycles of 15 s of denaturation at 94°C, 45 s of annealing at 55°C, and 30 s of extension at 72°C. The 18S rRNA gene was used as an endogenous control. At the end of the cycling phase, a dissociation curve was produced to ensure the specificity of the amplification. The relative expression ratios for each target gene were calculated using the 2- ΔΔCt method according to Livak and Schmittgen [72]. Details of the primers that were used and the genes that were tested are listed in Table 7.

Microscopy Analysis

Healthy and HLB affected round to triangular shaped young stems and roots showing primary and secondary growth were prepared for light and transmission electron microscopy using the following method. Samples were placed in 3% glutaraldehyde in 0.1 M potassium phosphate buffer, pH 7.2 for 4 hr at room temperature followed by 3 washes in the same buffer. Post-fixation in 2% osmium tetroxide in the same buffer was carried out for 4 hr at room temperature, then washed in two buffer changes before dehydration in acetone at 10% for 10 min each step. The samples were then infiltrated and embedded in Spurr’s resin [73]. One micrometer cross sections were made on an LKB Huxley Ultramicrotome (LKB Instruments Inc., Rockville, MD, USA) and stained with methylene blue/azure A, counter-stained with basic fuchsin [74]. These were photographed using a Leitz Labor-Lux S compound microscope (Leitz, Wetzlar, Germany) equipped with a Canon Power Shot S31 S digital camera. The same blocks were then thin sectioned with the same microtome, mounted on 200 mesh Formvar coated Cu grids followed by staining with 2% uranyl acetate and post staining with Reynolds lead citrate [75]. The grids were examined with a Morgagni 268 transmission electron microscope (FEI, The Netherlands) equipped with an AMT digital camera (Danvers, MA, USA).

Supporting Information

Figure S1 Cellular pathways that are regulated by Ca. L. asiaticus infection in the stems and roots of Valencia sweet orange (Citrus sinensis). A = stem and B = root. Genes that were significantly up-regulated following Ca. L. asiaticus infection are displayed in blue, and down-regulated genes are displayed in red.

(TIF)

Figure S2 Regulatory pathways that are altered by Ca. L. asiaticus infection in the stems and roots of Valencia sweet orange (Citrus sinensis). A = stem and B = root. Genes
that were significantly up-regulated following Ca. L. asiaticus infection are displayed in blue, and down-regulated genes are displayed in red. (TIF)

Figure S3 Starch and sugar metabolic pathway genes that are regulated by Ca. L. asiaticus infection in the stems of Valencia sweet orange (Citrus sinensis). Genes that were significantly up-regulated following Ca. L. asiaticus infection are displayed in blue, and down-regulated genes are displayed in red. Abbreviations/definitions: ADP-glucose pyrophosphorylase large subunit 3 (APL3), granule-bound starch synthase (GBSS), acid invertase (ACI), vacuolar invertase (VAI), alpha-amylose (AMY), beta-amylose (BMY), and sugar transporter 1 (SUT1). (TIF)

Figure S4 Regulation of transcription factor-encoding genes by Ca. L. asiaticus infection in the stems and roots of Valencia sweet orange (Citrus sinensis). A = stem and B = root. Genes that were significantly up-regulated following Ca. L. asiaticus infection are displayed in blue, and down-regulated genes are displayed in red. Abbreviations/definitions: ABI3/VP1, ABI3/VP1-related B3-domain-containing TF family; AP2/ EREBP, APETAL2/ethylene-responsive element binding protein family; ARF, auxin response factor; bZIP, basic leucine zipper motif; bHLH, basic helix-loop-helix family; C2C2-CO-like, CONSTANS-like zinc finger family; C2C2-DoF, C2C2/Zn DoF family; C2C2-YABBY, C2C2/Zn YABBY family; C2C2-GATA, C2C2/Zn GATA family; C2H2, C2H2 zinc finger family; C3H, C3H zinc finger family; CCAAT-DRI, CCAAT box binding factor DR1; ORPHAN, Orphan family; NAC, NAC domain; MYB-related, MYB-related family; MYB, MYB domain; MADS, MADS box domain; HB, homeobox TF family; HSF, heat shock TF family; GRF, GRF family; G2-like, G2-like family; GARP, GRAS, GRAS family; E2F-AP, E2F/DP family; EII, EIN3-like; CCAAT-HAP2, CCAAT box binding factor HAP2; CDP, CPP(Zn), CPP1-related family; SBP, SBP family; TCP, TCP domain TF; Global, Global TF group; High mobility, high mobility group (HMG) family; Trihelix, triple helix family; TUB, Tubby (TUB) homolog TF; Histone D1α, histone deacetylase; Histone H3, histone acetyltransferase; WRKY, WRKY domain family; AS2, lateral organ boundary gene family; JUMONJI, JUMONJI class TF; AT-rich, AT-rich interaction domain-containing family; AtSR, AtSR family; LUG, LEUNIG (LUG) domain family; Methyl BD, methyl binding domain proteins; Aux/1, Aux/1 family; B3, B5 DNA binding domain TF; NPR1, NPR1 family; NIN-like, NIN-like bZIP-related family; Bromodomain, bromodomain proteins; BZR, Brassinazole resistant TF family; Nucleosome assembly, nucleosome/chromatin assembly factor group; Chromatin Remodeling, chromatin remodeling factors; Phd finger, Phd finger family; PhOR1, photoperiod-responsive 1; Dicer-like, dicer; DNA MT, DNA methyltransferase; Polycomb, Polycomb group (PcG); Pseudo ARR, Pseudo ARR; ELF, ELF3 TF, FHA, Forkhead-associated (FHA) domain TF family; PWWP domain, PWWP domain protein family; SET-domain, SET-domain transcriptional regulator family; GFBP, GFBP-like family; General, general transcription; Silencing, silencing group; SNF7, SNF7 family; TAZ, Transcriptional co-activator with PDZ binding motif; CCHC, Zn-finger (CCHC). (TIF)

Figure S5 Regulation of secondary metabolic pathway genes by Ca. L. asiaticus infection in the stems and roots of Valencia sweet orange (Citrus sinensis). A = stem and B = root. Genes that were significantly up-regulated following Ca. L. asiaticus infection are displayed in blue, and down-regulated genes are displayed in red. (TIF)

Figure S6 Regulation of phenylpropanoid pathway genes by Ca. L. asiaticus infection in the stems of Valencia sweet orange (Citrus sinensis). Genes that were significantly up-regulated following Ca. L. asiaticus infection are displayed in blue, and down-regulated genes are displayed in red. There was no significantly up-regulated phenylpropanoid pathway genes observed in the roots. (TIF)

Author Contributions
Conceived and designed the experiments: VA NW. Performed the experiments: VA DA NW. Analyzed the data: VA DA NW FG GA. Contributed reagents/materials/analysis tools: VA DA NW FG GA. Wrote the paper: VA NW DA FG.

References
1. Bove JM (2006) Huanglongbing: a destructive, newly-emerging, century-old disease of citrus. J Plant Pathol 7–37.
2. Ogas T, Kohori Y, Kawasaki K, Yonemoto H, Ohno Y, et al. (2008) The effects of HLB-infection on respiration and development of roots of feronia rootstock (Feronia obtusa) which showed resistance to HLB bacterium. Gottwald TR, Graham JH (Eds) Proceedings of the 1st International Research Conference on Huanglongbing Orlando, FL, USA. pp. 184.
3. Johnson E, Bright DB, Graham JH (2012) Early root injury and damage in citrus huanglongbing disease development. Phytopathology 102: S4.59.
4. Kim JS, Sagaram US, Burns JK, Li JL, Wang N (2009) Response of sweet orange (Citrus sinensis) to 'Candidatus Liberibacter asiaticus' infection: microscopy and microarray analyses. Phytopathology 99: 50–57.
5. Achor DS, Exterberia E, Wang N, Folinominova SY, Chung KR, et al. (2010) Sequence of anatomical symptom observations in citrus affected with huanglongbing disease. Plant Pathol J 59: 56–64.
6. Exterberia E, Gonzalez P, Achor D, Albigo G (2009) Anatomical distribution of abnormally high levels of starch in HLB-affected Valencia orange trees. Physiol Mol Plant Pathol 74: 76–83.
7. Feng J, Chen C, Bransky RH, Gmitter FG, Li ZG (2010) Changes in carbohydrate metabolism in Citrus sinensis infected with 'Candidatus Liberibacter asiaticus'. Plant Pathol 59: 1037–1043.
8. Folinominova SY, Achor DS (2010) Early events of citrus greening (Huanglongbing) disease development at the ultrastructural level. Phytopathology 100: 949–956.
9. Baldwin E, Ploto A, Manthey J, McCollum G, Bai J, et al. (2010) Effect of liberibacter infection (huanglongbing disease) of citrus on orange fruit physiology and fruit/fruit juice quality: chemical and physical analyses. J Agric. Food Chem 58: 1247–1262.
10. Dagulo L, Dazyluk MD, Spann TM, Vath MF, Goodrich-Schneider R, et al. (2010) Chemical characterization of orange juice from trees infected with citrus greening (Huanglongbing). J Food Sci 75: C109–C207.
11. Rosales R, Burns JK (2011) Phytohormone changes and carbohydrate status in sweet orange fruit from Huanglongbing-infected trees. J Plant Growth Regul 30: 312–321.
12. Tatsnelli S, Sagaram US, Gowda S, Robertson CJ, Dawson WO, et al. (2008) In planta distribution of 'Candidatus Liberibacter asiaticus' as revealed by polymerase chain reaction (PCR) and real-time PCR. Phytopathology 98: 592–599.
13. Albrecht U, Bowman K (2008) Gene expression in Citrus sinensis (L.) Osbeck following infection with the bacterial pathogen 'Candidatus Liberibacter asiaticus' causing Huanglongbing in Florida. Plant Sci 143: 291–306.
14. Fan J, Chen C, Yu Q, Brlansky RH, Li ZG, et al. (2011) Comparative iTRAQ proteome and transcriptional analyses of sweet orange fruit infected with 'Candidatus Liberibacter asiaticus'. Physiol Plant 143: 235–245.
15. Martinelli F, Urasu SI, Albrecht U, Reagan RL, Phu ML, et al. (2012) Transcriptome profiling of citrus fruit response to huanglongbing disease. PLoS One 7: e30839.
16. Zheng ZL, Zhao Y (2013) Transcriptomic and gene coexpression network analysis provide a systems view of citrus response to 'Candidatus Liberibacter asiaticus' infection. BMC Genomics 14: 27.
17. Albrecht U, Bowman KD (2012) Transcriptional response of susceptible and tolerant citrus to infection with 'Candidatus Liberibacter asiaticus'. Plant Sci 185: 116–120.

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35. Gray-Mitsumune M, Mellerowicz EJ, Abe H, Schrader J, Winzell A, et al. (2004) 
34. Campbell P, Braam J (1999) Xyloglucan endotransglycosylases: diversity of 
33. Darley CP, Forrester AM, McQueen-Mason SJ (2001) The molecular basis of 
32. Lamport DT, Kieliszewski MJ, Chen Y, Cannon MC (2011) Role of the extensin 
29. Keegstra K, Walton J (2006) Plant science. Beta-glucans–brewer's bane, 
24. Thimm O, Blasing O, Gibon Y, Nagel A, Meyer S, et al. (2004) MAPMAN: a 
21. Martin JA, Wang Z (2011) Next-generation transcriptome assembly. Nat Rev 
41. Shuai B, Reynaga-Pena CG, Springer PS (2002) The lateral organ boundaries 
40. Verica JA, Chae L, Tong H, Ingmire P, He ZH (2003) Tissue-specific and 
39. Ryan CA, Pearce G (2003) Systemins: a functionally defined family of peptide 
37. Whitham S, McCormick S, Baker B (1996) The N gene of tobacco confers 
20. Kogenaru S, Qing Y, Guo Y, Wang N (2012) RNA-seq and microarray 
19. Liu P, Tammimies KP, Barry GF, Prene, J, Kishore GM (1992) Regulation of 
18. Somerville C (2000) Cellulose synthesis in higher plants. Annu Rev Cell Dev Biol 
17. Vogel J (2008) Unique aspects of the grass cell wall. Curr Opin Plant Biol 11: 301–307.
16. Keegstra K, Walton J (2006) Plant science. Beta-glucans–brewer's bane, 
15. Li M, Xiong G, Li R, Cui J, Tang D, et al. (2009) Rice cellulose synthase-like D4 
14. Thimm O, Blasing O, Gibon Y, Nagel A, Meyer S, et al. (2004) MAPMAN: a 
13. Lamport DT, Kieliszewski MJ, Chen Y, Cannon MC (2011) Role of the extensin 
12. Lamport DT, Kieliszewski MJ, Chen Y, Cannon MC (2011) Role of the extensin 
11. Davis SG, Tammimies KP, Barry GF, Prene J, Kishore GM (1992) Regulation of 
10. Zhou X, Ren L, Meng Q, Li Y, Yu Y, et al. (2010) The next-generation 
9. Li M, Xiong G, Li R, Cui J, Tang D, et al. (2009) Rice cellulose synthase-like D4 
8. Somerville C (2000) Cellulose synthesis in higher plants. Annu Rev Cell Dev Biol 
7. Thimm O, Blasing O, Gibon Y, Prene, J, Kishore GM (1992) Regulation of 
6. Lamport DT, Kieliszewski MJ, Chen Y, Cannon MC (2011) Role of the extensin 
5. Lamport DT, Kieliszewski MJ, Chen Y, Cannon MC (2011) Role of the extensin 
4. Lamport DT, Kieliszewski MJ, Chen Y, Cannon MC (2011) Role of the extensin 
3. Lamport DT, Kieliszewski MJ, Chen Y, Cannon MC (2011) Role of the extensin 
2. Lamport DT, Kieliszewski MJ, Chen Y, Cannon MC (2011) Role of the extensin 
1. Lamport DT, Kieliszewski MJ, Chen Y, Cannon MC (2011) Role of the extensin 
