Bacteriology

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Response of Sweet Orange (*Citrus sinensis*) to '*Candidatus* Liberibacter asiaticus' Infection: Microscopy and Microarray Analyses

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ABSTRACT

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Citrus greening or huanglongbing (HLB) is a devastating disease of citrus. HLB is associated with the phloem-limited fastidious prokaryotic α-proteobacterium '*Candidatus* Liberibacter spp.' In this report, we used sweet orange (*Citrus sinensis*) leaf tissue infected with '*Ca.* Liberibacter asiaticus' and compared this with healthy controls. Investigation of the host response was examined with citrus microarray hybridization based on 33,879 expressed sequence tag sequences from several citrus species and hybrids. The microarray analysis indicated that HLB infection significantly affected expression of 624 genes whose encoded proteins were categorized according to function. The categories included genes associated with sugar metabolism, plant defense, phytohormone, and cell wall metabolism, as well as 14 other gene categories. The anatomical analyses indicated that HLB bacterium infection caused phloem disruption, sucrose accumulation, and plugged sieve pores. The up-regulation of three key starch biosynthetic genes including ADP-glucose pyrophosphorylase, starch synthase, granule-bound starch synthase and starch debranching enzyme likely contributed to accumulation of starch in HLB-affected leaves. The HLB-associated phloem blockage resulted from the plugged sieve pores rather than the HLB bacterial aggregates since '*Ca.* Liberibacter asiaticus' does not form aggregate in citrus. The up-regulation of *pp2* gene is related to callose deposition to plug the sieve pores in HLB-affected plants.

Citrus greening or huanglongbing (HLB) is one of the most devastating diseases of citrus (5,11). The disease is associated with a phloem-limited fastidious α-proteobacterium, which has yet to be cultured. The HLB-associated bacterium was named '*Candidatus* Liberibacter spp.' based on its 16S rDNA sequence (20,29). Currently, three species of the pathogen, '*Ca.* Liberibacter asiaticus', '*Ca.* Liberibacter africanus', and '*Ca.* Liberibacter americanus', are recognized based on 16S rDNA sequence (4). '*Ca.* Liberibacter asiaticus' is the more prevalent species (4, 10,19,59–61). '*Ca.* Liberibacter americanus' is naturally transmitted to citrus by the psyllid *Diaphorina citri* Kuwayama and can be artificially transmitted by grafting from citrus to citrus and dodder (*Cuscuta campestris*) to periwinkle (*Catharanthus roseus*) or tobacco (*Nicotiana tabacum* Xanthi) (4). Typical symptoms of greening disease on leaves of infected citrus trees include reduced plant height, pale yellowing of leaves, blotchy mottle, and/or variegated chlorosis of leaves. Infected leaves can become upright, followed by leaf drop at the laminar abscission zone or petiole abscission and twig dieback at later stages (4). Early flowering is also observed in HLB pathogen-infected sweet orange. Previous studies have indicated that the HLB bacteria were unevenly distributed in phloem of bark tissue, and vascular tissue of the leaf midrib, roots, and different floral and fruit parts (49). The HLB bacterium has been reported to inhabit living phloem cells, a different environment from foliar and intercellular spaces, and avoids the extracellular surface receptors encountered by many pathogens.

Infection of plants by bacteria, in general, causes extensive changes of gene expression involved in plant defense, environmental stress response metabolism, protein metabolism, transport, energy, and others (18,50,51,58,64). Up to 25% of the total *Arabidopsis* transcriptome was affected by pathogen infection (39,58). Affected gene expressions represent compatible or incompatible interactions between the host and pathogen. Plants have evolved multiple defense mechanisms in response to pathogen attack, and the pathogens have evolved multiple counter measures to host defenses (25). Plant defense responses in incompatible responses include the hypersensitive response resulting in localized cell death, structural alterations, and production of plant defense molecules such as antimicrobial proteins (7,23). At the same time, little is known about the molecular basis of the plant response to virulent pathogens in compatible plant–microbe interactions. Suppression of host defenses, including basal defenses, gene-for-gene resistance, and nonhost resistance, is critical for pathogenesis as shown with susceptible plant–*Pseudomonas syringae* interactions (43). *P. syringae* pv. *tomato* was also recently reported to influence the abscisic acid signaling pathway of *Arabidopsis* sp. to cause disease (12).

We are analyzing the host response to HLB pathogen infection of phloem tissue. Despite the many visual and physiological observations on HLB-affected citrus plants worldwide, the molecular determinants for the HLB disease have yet to be established. Phloem is an ideal habitat for more than 12 disease agents including *Phytoplasma* spp., *Spiroplasma* spp., and '*Ca.*

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*The *e*-Xtra logo stands for "electronic extra" and indicates that the online version contains a table showing differentially regulated sweet orange genes in response to HLB pathogen infection.

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Liberibacter spp.' (4,6,36) due to the presence of rich nutrients in phloem sap. Many of those disease-causing organisms inhabiting phloem are thought to reside in those tissues exclusively or nearly so. Consequently, phloem-limited bacteria might induce disease by physically plugging the phloem in some way and affecting transportation of metabolites. For example, phytoplasma infection affects phloem function by inducing callose deposition at sieve plates, eventually causing necrosis and collapse of sieve elements thereby altering content of phloem sap (36). Similarly, host anatomical aberrations including necrotic phloem, massive accumulation of starch, and disordered cambial tissue were observed in sweet orange with typical HLB symptoms from South Africa (52), although presence of the HLB pathogen in the phloem was not confirmed. These reports need to be verified because new information has come to light indicating that a *Phytoplasma* sp. can cause very similar symptoms of HLB in citrus in Brazil (62). It is in this light that more information on the relationship between '*Ca.* Liberibacter asiaticus', symptom development, and host gene response is needed.

Here, changes in host gene expression to the HLB infection were assessed using microarray analyses of 33,879 expressed sequence tag (EST) collections. We also present the resulting changes in tissue morphology by comparing uninfected host leaves with leaves infected by '*Ca.* Liberibacter asiaticus' using light and transmission electron microscopy.

MATERIALS AND METHODS

Plant materials. Young, healthy, sweet orange plants were graft-inoculated with budwood from HLB-affected sweet orange trees from the field and kept in an U.S. Department of Agriculture-APHIS/CDC-approved, secured greenhouse at the Citrus Research and Education Center, University of Florida, Lake Alfred, FL. This HLB pathogen inoculum source has been carefully selected. Phloem tissues from several citrus groves with HLB were tested for *Citrus tristeza virus* (CTV), *Xanthomonas* spp., and other common pathogens in Florida. Branches from one new citrus grove were free of CTV and other pathogens and were used as the inoculum in the greenhouse. We later conducted the bacterial diversity study of the phloem tissues of the same plants in the greenhouse by constructing the 16S rDNA library. Only '*Ca.* Liberibacter asiaticus' was found in the phloem tissues of the inoculated plants used for microarray analysis in the greenhouse (N. Wang, *unpublished data*). In total, eight plants were inoculated by grafting. Yellowing symptoms were shown 4 months after inoculation from five plants. Characteristic mottle symptoms were shown about 7 months after inoculation. In order to eliminate the potential effect due to tree size, three infected trees showing similar symptoms with similar size to healthy control were chosen for this study. Symptomatic leaf samples were collected 8 months after inoculation from HLB-affected sweet orange (*Citrus sinensis*). As a control, leaf samples at the same developmental stage were collected from healthy control plants. Three samples from three HLB-infected plants and two healthy plants were collected, respectively. Presence of the HLB bacterium was confirmed with polymerase chain reaction (PCR) analysis with primers A2/J5 (26) and quantitative PCR (Q-PCR) with primer/probe set CQULA04F-CQULAP10-CQULA04R (63).

DNA microarray analysis. Total RNA was extracted from each sample using the RNeasy plant kit (Qiagen, Valencia, CA). Samples were collected 8 months after inoculation. Three replicate HLB-affected and healthy plants were used. In order to eliminate variation between different plants, leaves at similar growth stages were collected from infected and healthy control replicate plants. Samples were divided into three tubes for RNA extraction after grinding in liquid nitrogen. RNA concentration was determined with a NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE), and sample quality was assessed with the Agilent Model 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Four micrograms of total RNA was processed for use on the GeneChip microarray by the Affymetrix GeneChip one-cycle target labeling kit (Affymetrix, Santa Clara, CA) according to the manufacturer's recommended protocols. The resultant biotinylated cRNA was fragmented and then hybridized to the GeneChip Citrus Genome Array (30,171 probe sets representing up to 33,879 citrus transcripts based on EST sequences obtained from several citrus species and citrus hybrids; Affymetrix). The arrays were washed, stained, and scanned using the Affymetrix Model 450 Fluidics Station and Affymetrix Model 3000 7G scanner according to the manufacturer's recommended protocols. Microarray experiments were performed at the Interdisciplinary Center for Biotechnology Research Microarray Core, University of Florida.

Data analysis. Statistical tests were performed using the BioConductor statistical software (available online). This is an open source and open development software project for analysis of microarray and other high-throughput data based primarily on the R programming language (21). The raw data were normalized by robust multichip analysis approach implemented in Affy package (3). A linear modeling approach and the empirical Bayes statistics as implemented in the limma package (54) in the R software were employed for differential expression analysis. Differentially expressed genes were ranked by P values, and genes with P value of ≤ 0.05 were considered differentially expressed genes at a statistically significant level.

Quantitative reverse transcription-PCR (QRT-PCR) analysis. All QRT-PCR reactions were performed in a 25-µl reaction in an Applied Biosystems 7500 Fast Real-Time PCR system (Foster City, CA) with the QuantiTect SYBR Green RT-PCR kit (Qiagen) using 0.4 µM of each primer and 50 ng of RNA template. 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, 30 s of annealing at 55°C, and 30 s of extension at 72°C. The 18S rRNA gene expression was used as an internal control. The following primers for QRT-PCR analysis were designed using Primer 3 (48): B292132: GCCAAAGCTTGAGTACCATAGG/CTGTGGAAGAAGGCTTTACAGG; CX070113: AAATTAGCAGCAGATGTTCCAG/TCCCACGATTCTATTTTGCTTC; CX639454: AATGGAAACAGCATCTCACAAG/TTGAAGATATGCATCGACAACC; DT214451: AACAAAGCCGCAAGTATACCAC/TGAGGAACTTATGGAAGCAACC; CF653559: AAATGTGGGTGAATGAGAAAGC/ATTATTGTTGCACGTCACCTTG; CK935883: AAATACAATGGCAGCAGCATC/AACCTCTTGCAAACCTGAAAAG; CX045772: GATCCACTGCCTCAAGACTAGG/AAGCTGAGGGTCCTAGAGAAGC; CX076036: TGCTCACTCACACTCAGACAAC/AAAATCGGATGACGTGTCTCTC; and 18S: GTGACGGAGAATTAGGGTTCG/CTGCCTTCCTTGGATGTGGTA.

Microscopy. Midribs from HLB-affected and healthy leaves were cut into 2- to 3-mm segments. For light microscopy (LM) and transmission electron microscopy (TEM), tissues were fixed in 3% glutaraldehyde in 0.1 M potassium phosphate buffer, pH 7.2, overnight at 4°C. Samples were washed in the same buffer and postfixed in 2% osmium tetroxide for 4 h at room temperature and then dehydrated in acetone and embedded in Spurr's resin (55). For LM, 1-µm sections were made on an ultramicrotome (Huxley; LKB Instruments Inc., Rockville, MD) with glass knives and stained with methylene blue-azure A and basic fuchsin (27,53). Light micrographs were made using a standard research light micrograph (Carl Zeiss, West Germany) with an attached camera. For TEM, thin sections (90- to 100-nm) were made with a diamond knife on the same microtome, mounted on copper grids, and stained with uranylacetate (56) and lead citrate (46). Samples were examined in an FEI Morgagni 268 TEM, and images were captured and analyzed with Image-Pro software. For aniline blue staining, samples were cut into 2- to 3-mm sections

and infiltrated with Tissue-Tek OCT medium and then mounted on specimen pins designed to fit a Harris cryomicrotome. Samples were frozen in the same chamber, sectioned, and then stained with 0.05% aniline blue in 0.1 M phosphate buffer (pH 8.5) for 5 min (9). Specimens were examined by epifluorescence optics with a Zeiss AX 10 fluorescence microscope under UV illumination provided by an HBO 100 mercury arc lamp.

Sucrose assays. For extraction of sucrose, leaf samples collected randomly from the same set of samples for microarray analysis including '*Ca.* Liberibacter asiaticus' infected and healthy plants were frozen and ground in liquid nitrogen. Sugars were extracted using 80% (vol/vol) ethanol (Acros, NJ) as described by Mohammed et al. (41) with modifications. Briefly, 0.07 g of pulverized leaf material was submerged into 1.5 ml of ethanol and incubated at 60°C for 2.5 h with frequent mixing of the plant material by inverting the tubes several times. The tubes were spun at 14,000 rpm for 1 min and the supernatant was collected. The extraction steps were repeated three times, and the extracts were pooled and the supernatant discarded. The pooled extract (7 ml) was treated with activated charcoal (Sigma-Aldrich, St. Louis, MO) to absorb phenols and chlorophylls that might interfere with enzymes for sugar analysis. The extracts were stored either as ethanol or as aqueous solution (after evaporation of ethanol and dissolving with water) at –20°C until further analysis. Aliquots of aqueous solutions were assayed for sucrose using an enzymatic assay kit (SCA-20) from Sigma. The sugars were quantified through reduction of nicotinamide adenine dinucleotide (NAD) to NADH and measured as absorbance at 340 nm using a Benchmark Plus microplate spectrophotometer (Bio-Rad Laboratories, Hercules, CA).

RESULTS AND DISCUSSION

Microarray analysis identified numerous categories of host metabolism affected by HLB pathogen infection. The citrus microarrays used for this work contains up to 33,879 citrus transcripts from several citrus species and citrus hybrids. As such, it is likely that the entire *Citrus sinensis* transcriptome is not fully represented on the array. Despite this, these arrays remain the most powerful tool to date for exploring global citrus gene expression. To identify differential gene expression between HLB-affected and control plants, two criteria were applied. First, differentially expressed genes were ranked by *P* values; genes with *P* values of ≤0.05 were considered differentially expressed genes. From this pool of transcripts, genes were considered up- or down-regulated if the log2 ratio of infected to healthy hybridization results were greater (positive) than or less than (negative) 1.5-fold, respectively. A total of 624 genes were significantly regulated: 307 genes were up-regulated and 317 genes were down-regulated in infected trees. The host genes affected by HLB bacterium infection were related to plant pathogenesis/stress (10.4% of the total), anthocyanin biosynthesis (0.5%), cell wall metabolism (6.6%), cell division (0.96%), detoxification (6.2%), lipid metabolism (2.4%), metabolite transport (5.8%), metal transport (2.6%), nucleotide metabolism (1.6%), phenylpropanoid/flavonoid/terpenoid metabolism (5.6%), phytohormones (3.0%), protein kinase (5.0%), protein metabolism (3.8%), protein–protein interaction (1.1%), signal transduction (1.8%), sugar metabolism (4.3%), transcription/translation factors (7.2%), and unknown/hypothetical genes (31.1%) (Fig. 1; Tables 1 and 2). The broad range of host genes affected by HLB infection suggested profound disturbances in plant metabolism. Some categories of genes were mostly down-regulated in infected trees; these were genes related to cell cycle, cell wall metabolism, lipid metabolism, nucleotide metabolism, and protein kinase, whereas genes associated with metal transport were mostly up-regulated. Differential expression of nine genes was tested by QRT-PCR using the same RNA preparations used for microarray analysis (Table 3). Primers designed from 18S rDNA were used as internal control for normalization. Expression of the eight genes examined with QRT-PCR paralleled that observed by microarray analysis, albeit the changes were not quantitatively identical as reported previously (1,18,38). The *pp2* gene was highly up-regulated in HLB-affected leaves based on microarray analysis, but QRT-PCR data indicated a much lower induction. Further investigation is in the process to verify *pp2* and other interesting genes.

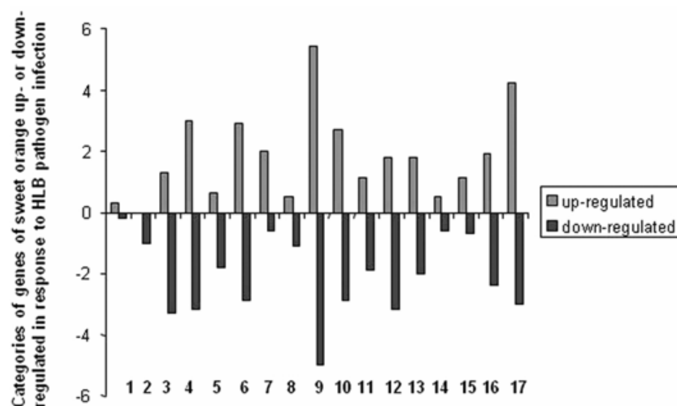

Fig. 1. Classification of sweet orange (*Citrus sinensis*) huanglongbing (HLB)-regulated genes into functional categories in response to infection with '*Candidatus* Liberibacter asiaticus'. Light bar represents genes up-regulated in response to the HLB pathogen infection. Dark bar represents genes down-regulated in response to HLB pathogen infection. Different gene categories are indicated by different numbers: 1, anthocyanin biosynthesis; 2, cell cycle; 3, cell wall protein; 4, detoxification; 5, lipid metabolism; 6, metabolite transport; 7, metal transport; 8, nucleotide metabolism; 9, pathogenesis-related and stress-related; 10, phenylpropanoid/flavonoid/terpenoid; 11, metabolism; 12, phytohormone-related protein kinase; 13, protein metabolism; 14, protein–protein interaction; 15, signal transduction; 16, sugar metabolism; and 17, transcription/translation factors.

Over 10% of the genes significantly regulated in plants infected with '*Ca.* Liberibacter asiaticus' were related to plant defense and stress. Among these were genes whose encoded products were classified as pathogenesis-related (PR) proteins such as chitinase, PR-1 precursor, disease resistance-responsive protein, blight-associated protein p12 precursor, disease-resistance protein, PR protein 4A, and Avr9 Cf-9 elicited protein 111B. Approximately half of the PR genes were up-regulated by '*Ca.* Liberibacter asiaticus' infection. Interestingly, one gene encoding blight-associated protein P12 precursor was up-regulated. The function of P12 is still unknown and has been suggested to play a role in host response to citrus blight (13). The up-regulation of PR genes in the host may be an indication of activation of defense mechanisms that lead to processes such as callose deposition in and around phloem tissues (2). Several defense-associated transcription factors that bind to promoter elements of individual defense-related genes were also up-regulated including WRKY4, WRKY6 (15,17,32), ERF-1, ERF-2 (24), TGA (28,35), and R2R3-MYB (34,57). Approximately half of the PR genes were down-regulated by '*Ca.* Liberibacter asiaticus' infection. Numerous PR genes were also up- or down-regulated in both virulent and avirulent *Pseudomonas syringae* infections in *Arabidopsis* spp. (58). It has been suggested that PR genes are up-regulated in a more intense and/or accelerated manner during the incompatible interaction (39). Suppression of host defenses including basal defense, gene-for-gene resistance, and nonhost resistance was shown to be critical for pathogenesis (43). Thus, our work supports the idea that up- and down-regulation of PR genes indicates that the host has reacted to invasion and infection by the HLB pathogen in a pattern that has been reported by others.

Over 4% of the genes significantly regulated in plants infected with '*Ca.* Liberibacter asiaticus' were genes related to sugar metabolism such as starch synthesis and degradation. In plants,

four major enzymes control starch biosynthesis: ADP-glucose pyrophosphorylase (AGPase), starch synthase, granule-bound starch synthase, and starch debranching enzyme. The rate-limiting enzyme is AGPase, which converts glucose-phosphate to ADP-glucose in the presence of ATP. ADP-glucose then is polymerized into α-(1,4)-linked chains (α-amylose) by multiple isoforms of starch synthase. Granule-bound starch synthase catalyzes the addition of glucose units to form an essentially linear polymer of α-amylose with very few branches (42). Branching of amylopectin is the result of the balanced activities of starch-branching enzymes and starch-debranching enzymes. Three of the four starch synthesis genes including AGPase, starch synthase, and granule-bound starch synthase were up-regulated in HLB-affected citrus leaves (Table 2). Interestingly, genes directly associated with photosynthesis were not influenced by HLB pathogen infection. The up-regulation of key starch biosynthetic genes with photosynthesis apparently proceeding unaltered, together with restricted movement of photosynthates from leaves due to phloem plugging, likely lead to accumulation of starch in HLB-affected leaves (Fig. 2).

TABLE 1. Huanglongbing regulation of pathogenesis- and stress-related genes[a]

Public ID	Annotation	Ratio of gene expression
CX668300	Chitinase	4.04
CF832155	Acidic class II chitinase	3.90
CX638776	Delta 1-pyrroline-5-carboxylate synthetase	3.48
CK937251	Miraculin-like protein	3.29
CX303148	Nam-like protein 11	3.07
CN186431	CTV protein	2.79
CX301461	Unnamed protein product	2.76
DN619110	Unknown protein	2.76
CX669483	Early nodulin	2.73
DN618893	Tyrosine aminotransferase	2.68
CX306211	Nectarin 5	2.62
CF507855	Delta 1-pyrroline-5-carboxylate synthetase 2	2.50
CF653559	Pathogenesis-related protein PR-1 precursor	2.44
CK935794	Glycolate oxidase	2.37
CX666928	BURP domain-containing protein	2.36
CX305834	Seed-specific protein	2.33
CX640129	Glutaredoxin-like protein	2.31
CK935883	Disease resistance-responsive protein	2.23
CX045772	Putative cell death associated protein	2.19
CN191283	NAM-like protein	1.97
CK934775	Disease-resistance protein	1.95
CX286941	Nodulin-like	1.95
CX301618	Dehydration-responsive protein RD22	1.76
CX296222	Blight-associated protein p12 precursor	1.71
CX641603	Disease-resistance protein	1.70
DR406181	Metallothionein-like protein	1.69
CB293886	NAM-like protein	1.68
CF835337	Pathogenesis-related protein 4A	1.65
CX048331	Putative ripening-related protein	1.65
CX637285	Pathogenesis-related protein 4A	1.63
CX044399	NBS-LRR resistance-like protein RGC359	1.62
DN618428	BURP-domain containing protein	1.61
CX545242	Berberine bridge enzyme-like protein	1.60
AU186381	Acidic class II chitinase	1.57
DN958104	Beta-cyanoalanine synthase	–1.50
CX076066	Resistance protein candidate RGC2J	–1.51
CF835944	MLO protein (mildew resistance locus)	–1.51
CX671223	Chitinase	–1.62
AU300664	Pathogenesis-related protein	–1.62
CF832471	Multi-copper oxidase	–1.62
DN795254	Putative calmodulin-binding protein	–1.68
CX292843	Photoassimilate-responsive protein	–1.70
CF507442	Nodulin-like protein	–1.71
CX305678	Basic chitinase	–1.72
CX048700	Leucine-rich repeat protein	–1.73
DN625052	Disease resistance RGA3 protein	–1.79
CX070975	Small MW heat shock protein	–1.84
CX671683	Nodulin-like protein	–1.90
DN618117	NBS-LRR type disease resistance protein	–1.91
CX045895	Chitinase	–1.91
CX291159	Elicitor-inducible cytochrome P450	–2.05
CF833037	Putative salicylate monooxygenase	–2.06
CF829440	Pectate lyase	–2.07
CV709277	Phosphoesterase family protein	–2.08
CX069929	Abl interactor-like protein-1	–2.12
CX643843	Putative BURP domain containing protein	–2.14
CX293287	Putative nodulin protein	–2.18
CO913159	Glyoxal oxidase related	–2.34
CF838393	Chitinase class II precursor	–2.44
CX637639	Chitinase	–2.44
CK936056	Subtilisin-like protease	–2.53
CF417485	ENSP-like protein	–2.63
CX296119	Immediate-early fungal elicitor protein CMPG1	–2.89
CN181971	Disease resistance LRR family protein	–3.06
CX076036	Avr9 Cf-9 rapidly elicited protein 111B	–4.58

[a] Ratio of gene expression represents log2 of infected versus uninfected samples based on microarray analysis.

TABLE 2. Huanglongbing regulation of genes related to sugar metabolism[a]

Public ID	Annotation	Ratio of gene expression
CX303072	Beta-glucosidase-like	3.98
DN622894	ADP-glucose pyrophosphorylase	3.53
DN625620	Glucose-6-phosphate dehydrogenase	2.72
CB292132	Granule-gound starch synthase	2.71
CX637561	Putative UDP-glucuronosyltransferase	2.64
DT214451	Beta-amylase	2.56
CX046632	Extracellular acid invertase 1	2.52
CX070113	Starch branching enzyme	2.20
CB292174	Sugar transport protein	1.89
CX045485	Hexose transporter	1.70
CX665157	Galactose oxidase	1.68
CX639454	Plant glycogenin-like starch initiation protein	1.62
CX663848	Glycosyl transferase	–1.52
CV705038	4-alpha-galacturonosyltransferase	–1.55
CF831824	Trehalose-phosphatase	–1.59
CK938541	Galacturonosyltransferase	–1.60
CX294095	Glycosyl hydrolase	–1.64
CN187456	Trehalose-phosphatase	–1.77
CK938256	Mannase	–1.84
CV886325	Glycerol-3-phosphate dehydrogenase	–1.89
DN617689	Raffinose synthase	–2.03
CF836851	Probable alcohol dehydrogenase	–2.40
DN959139	Trehalose-6-phosphate phosphatase	–2.40
CX044393	Probable short chain alcohol dehydrogenase CPRD12	–2.45
CK936380	Alcohol acyl transferase	–2.86
BQ623570	Dirigent protein	–3.13
CX309407	Alpha-glucosidase-like	-4.74

[a] Ratio of gene expression represents log2 of infected versus uninfected samples based on microarray analysis.

TABLE 3. Quantitative reverse transcription-polymerase chain reaction (QRT-PCR) assays of representative genes for the validation of microarray analysis[a]

EST	Predicted protein	QRT-PCR	Microarray
CB292132	Granule-bound starch synthase	1.83 ± 0.16	2.71
CX070113	Starch branching enzyme	1.56 ± 0.21	2.20
CX639454	Plant glycogenin-like starch initiation protein	1.41 ± 0.27	1.62
DT214451	β-Amylase	2.97 ± 0.24	2.56
CF653559	Pathogenesis-related protein PR-1 precursor	1.93 ± 0.07	2.44
CK935883	Disease resistance-responsive protein	2.28 ± 0.12	2.23
CX045772	Putative cell death associated protein	1.59 ± 0.10	2.29
CX076036	Avr9 Cf-9 rapidly elicited protein 111B	0.21 ± 0.01	–4.58

[a] Data represent the mean ±SD fold differences in gene expression in symptomatic leaves compared with nonsymptomatic leaves. QRT-PCR analysis of gene expression was performed with SYBR-Green as the fluorescent reporter. The expression of each gene was normalized to endogenous 18S rRNA. The gene expression was calculated using $2^{-\Delta\Delta Ct}$ (37) method. Each value of QRT-PCR is the mean of three biological × three technical = nine replicates.

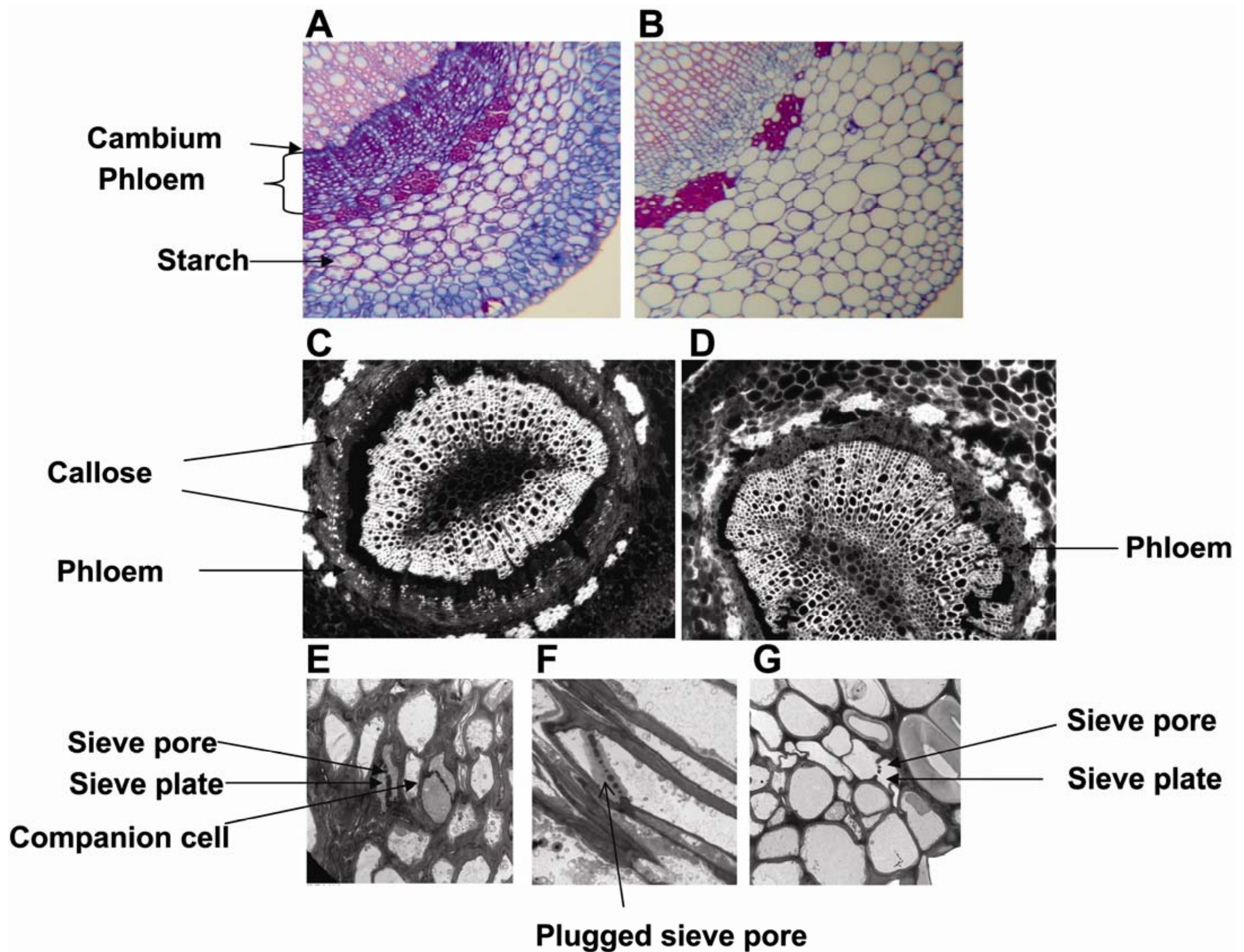

Fig. 2. Anatomical analysis of midrib phloem tissues of the huanglongbing (HLB) affected and healthy sweet orange. **A and B** show the methylene blue-azure A and basic fuchsin staining. Staining shows **A,** thickened and disrupted cell walls of phloem tissues of affected citrus compared with **B,** the healthy control. Starch particles stained red are also observed in the mesophyll parenchyma cells of the HLB-affected plant only. **C** and **D** show the callose staining of citrus midrib in 0.05% aniline blue solution observed under a fluorescent microscope with UV filter. Light areas reveal callose in the phloem. **C,** The infected midrib is full of callose in the phloem tissue, while **D,** the healthy control does not show light staining of callose. **E, F, and G,** Transmission electron microscopy of phloem tissue. **E and F,** Cell walls are thicker and disrupted and sieve pores are plugged in the HLB-affected plant while not in the healthy plant (**G**).

A total of 19 phytohormone-related genes were regulated in HLB-affected plants. These included genes encoding products associated with metabolism and function of gibberellic acid (8), auxin (5), cytokinin (3), ethylene (2), and ABA (1). Phytohormones play important roles in plant development, plant defense, and signal transduction. The imbalance of phytohormones might contribute to HLB symptom development such as early flowering, leaf and fruit deformities, and seed abortion. Salicylic acid (SA) and jasmonates (JA), critical in plant defense (47), might not have a prominent role in the host response because SA- and JA-related genes in the arrays were not significantly regulated by HLB infection.

The mechanism of sieve pore plugging involves PP1, PP2, and callose (14,33). PP2, a dimeric poly-GlcNAc-binding lectin, covalently cross-links with PP1 via disulphide bonds, forming polymers that close sieve pores (45). This response is normally accompanied by the synthesis of the beta-1,3-glucan callose (40). The *pp2* gene was highly up-regulated in HLB-affected leaves based on microarray analysis even though QRT-PCR data indicated a much lower induction. PP2 protein likely participated in phloem blockage together with callose (Fig. 2). Additionally, PP2 has been suggested to interact with a variety of putative signaling RNAs (22,30,31,44) and as such may interfere with communication between source and sink organs and nutrient transport.

HLB infection results in phloem damage, plugging of sieve pores, and interference with sucrose transport. PCR analysis of leaves from which midribs were excised using primers A2 and J5 (2) targeting the β-operon region of '*Ca.* Liberibacter asiaticus' to confirm the presence of '*Ca.* Liberibacter asiaticus' in infected leaves and the absence of bacteria in uninfected leaves (data not shown). The midribs of uninfected and HLB-affected sweet orange leaves were stained with the general polychromatic/complex carbohydrate staining procedure using methylene blue-azure A and basic fuschin (53) and observed with LM. The phloem cell wall and cambium layer of infected leaves were thicker than uninfected control leaves. Accumulation of starch was observed in phloem parenchyma cells of infected leaves but not of healthy leaves (Fig. 2A and B). Quantitative study of the starch indicated that its concentration in the HLB-affected leaves average 8.5 time higher than in the healthy leaves of sweet orange (E. Etxeberria, *personal communication*). This is consistent with the up-regulation of three major starch synthesis genes (Table 2). Aniline blue was used as a specific dye for callose deposition in infected and healthy midribs (9). Callose deposition was observed

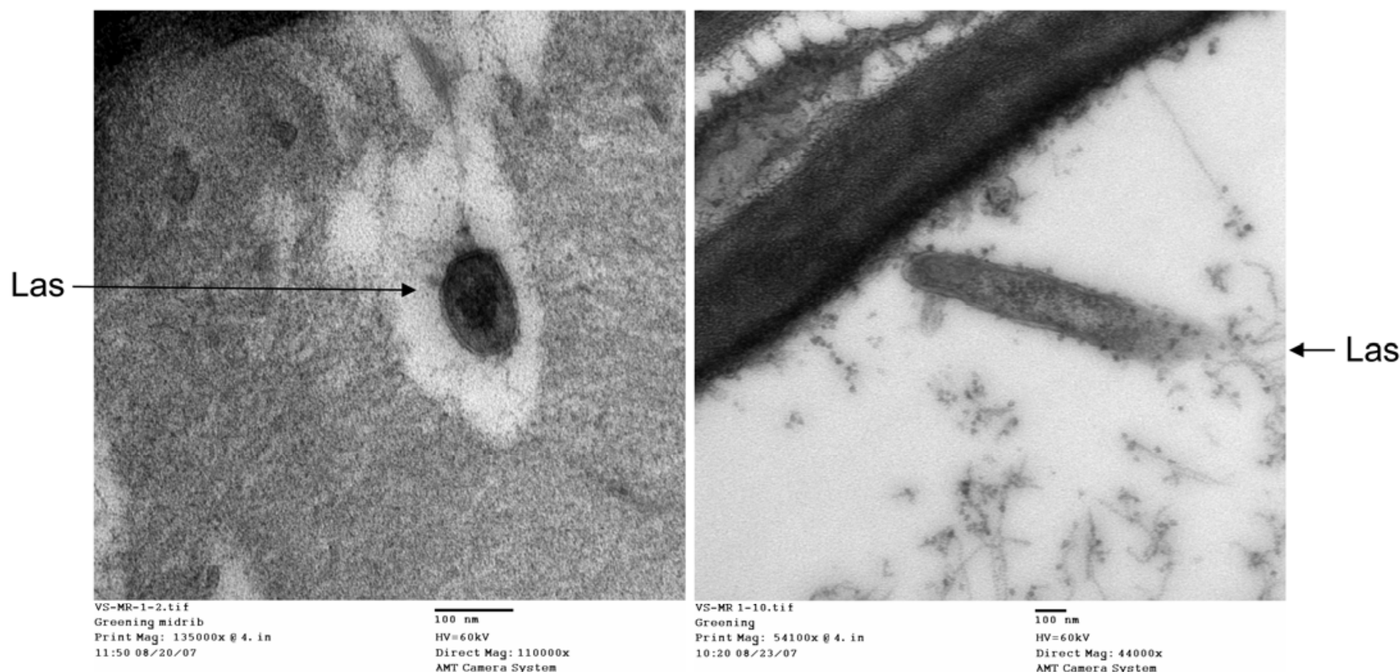

Fig. 3. '*Candidatus* Liberibacter asiaticus' in sweet orange midrib observed under transmission electron microscopy. Left: cross section; right: longitudinal section.

in infected but not healthy midribs (Fig. 2C and D). TEM observation of the infected midrib indicated that sieve pores of the infected plant were plugged with an amorphous substance. Collapse of sieve tubes and companion cells were also observed in HLB-affected midribs and not observed in healthy midribs (Fig. 2E, F, and G). These anatomical changes in sweet orange associated with '*Ca.* Liberibacter asiaticus' are similar to previous reports by Schneider (52) which was probably due to '*Ca.* Liberibacter africanus' infection. Phloem blockage was partially due to the deposition of large amount of callose as confirmed by staining with aniline blue (Fig. 2).

Sucrose is the major photoassimilate transported in sieve tubes from mature leaves to sink organs such as young leaves, flowers, roots, and fruits (6,65). In order to characterize the effect of phloem blockage on photoassimilate transport, sucrose assays were performed to compare sucrose concentrations in leaves of healthy and HLB-affected plants. The assays revealed that sucrose levels were higher (11.51 ± 1.81 mg/g) in HLB-affected leaves compared with healthy leaves (8.47 ± 1.81 mg/g). The accumulation of sucrose in the HLB-affected leaves, likely a result of phloem blockage, causes accumulation of starch. As the duration of blockage increases, nutrient deficiency in sink organs and hindrance of plant growth, fruit maturation, and seed development will be likely consequences.

In order to investigate whether '*Ca.* Liberibacter asiaticus' itself could block sieve pores, midribs from the HLB-affected citrus leaves were collected for TEM analysis. Of all midrib samples observed by TEM, '*Ca.* Liberibacter asiaticus' existed as single cells when present and did not form visible aggregates in the phloem (Fig. 3). Furthermore, no plug composed of '*Ca.* Liberibacter asiaticus' was observed in phloem sieve pores. Given the size of '*Ca.* Liberibacter asiaticus', it is unlikely that a single HLB bacterium could plug the sieve pore since the bacterium is about 2 μm long and 0.1 to 0.2 μm in diameter (4) while the pores on the sieve plates range from less than 1 μm to about 14 μm (16). Furthermore, our TEM observations also indicated that the HLB bacterium can pass through the sieve plate pore (data not shown). Consequently, it is unlikely that the HLB bacterium physically caused phloem blockage because multiple bacterial cells were not aggregating; rather, it is likely that the host response results in sieve pore plugging. In conclusion, we have shown that the HLB pathogen alters host gene expression which leads to symptom development. Future work will focus on the role of select genes in affected metabolic categories in symptom development.

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Erratum

William O. Dawson has been added to the acknowledgment section. Changes to this article were made on 5 December 2012.