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Citrus Huanglongbing

Nian Wang[1,2*] • Wenbin Li[3] • Mike Irey[4] • Gene Albrigo[1] • Kong Bo[5] • Jeong-soon Kim[1]

[1] Citrus Research and Education Center, University of Florida, 700 Experiment Station Road Lake Alfred, FL 33850, USA
[2] Department of Microbiology and Cell Science, University of Florida, Gainesville, FL 32611, USA
[3] National Plant Germplasm and Biotechnology Laboratory USDA -APHIS-PPQ-CPHST BARC-East, Powder Mill Road, Bldg 580 Beltsville, MD 20705, USA
[4] United States Sugar Corporation 111 Ponce de Leon Avenue Clewiston, Fl 33440, USA
[5] Taian College, Taian, Shandong, China

Corresponding author: * **nianwang@ufl.edu**

ABSTRACT

Citrus Huanglongbing is one of the most devastating diseases of citrus. This disease is associated with a phloem-limited, non-cultured, Gram-negative alpha-proteobacteria: *Candidatus* Liberibacter spp. The pathogen causes blotchy mottle on the leaves, yellow shoots, stunted growth, tree decline, as well as lop-sided fruits. In this review, we attempt to present a compilation of the current research activities on the diagnosis, *in planta* distribution of the bacterium and symptomology, and host response to HLB pathogen infection.

Keywords: Citrus greening, virulence mechanism

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INTRODUCTION

Citrus Huanglongbing (HLB) has attracted tremendous attention due to the recent spread of the disease in two major citrus production areas: Brazil and the United States. Several excellent reviews have been published on the history, worldwide distribution, symptomology, psyllid transmission, as well as epidemiology of citrus HLB. The reader is referred to the following detailed studies: da Graça (1991), Halbert and Manjunath (2004), Bové (2006) and Gottwald *et al.* (2007). In this review, we attempt to present a compilation of the current research activities on the diagnosis, *in planta* distribution of the bacterium and symptomology, and host response to HLB infection.

HLB, also referred to as citrus greening, is one of the most destructive diseases of citrus, debilitating the productive capacity of citrus trees (Bové *et al.* 1974; da Graça and Korsten 2004), with losses of 30-100% reported (Schwarz 1967; Aubert 1993). The disease is widespread in most citrus areas of Asian countries, Africa, Brazil and most recently, Florida and Louisiana. HLB was found in Florida in 2005 and has spread throughout thirty of the citrus producing counties in Florida by January 2008. The newest discovery was in Louisiana, which was reported in June 2008. To date, HLB has not been found in other citrus growing regions in the U.S. e.g., Texas, California, and Arizona. However, *Diaphorina citri* Kuwayama, the vector of *Candidatus* Liberibacter asiaticus (Las), was reported in Texas in 2001 and had rapidly spread throughout the state by 2006. Psyllids have also recently been found in Tijuana, Mexico at the California border. With the vector present, the potential of HLB spread to other citrus production states is high (French *et al.* 2001; da Graça *et al.* 2006).

Circumstantial evidence indicates that HLB is caused by a fastidious phloem-limited α-proteobacteria in the genus, *Candidatus* Liberibacter, although Koch's postulates have not been completed due to the difficulty in culturing the bacterium. *Ca*. Liberibacter spp. are Gram negative based on evidence from electron micrographs, inhibition by a cell wall disrupting antibiotic, penicillin, as well as sequence analysis based on 16S rDNA, the β-operon and other limited DNA sequence information (Bové 2006). Three species of HLB-causing bacteria have been identified as *Ca.* L. asiaticus (Las), *Ca.* L. americanus (Lam), and *Ca.* L. africanus (Laf) that differ in their 16S rDNA sequence, and environmental tolerances. Among them, Las is the most wide-spread and virulent strain. A recent study by Lopes *et al.* indicated that a shift in species prevalence was observed in the citrus groves of Brazil over time, with an increase of Las and decrease of Lam (S. Lopes, pers. comm.).

The citrus HLB bacteria are vectored by either *D. citri* or *Trioza erytreae* Del Guercio (McClean and Oberholzer 1965; Capoor *et al.* 1967; Bové 2006).

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Invited Mini-Review

DIAGNOSIS OF CITRUS HLB

The current management strategy for the control HLB is to remove infected citrus trees and reduce psyllid populations with insecticides. This strategy requires sensitive and reliable diagnostic methods.

Symptoms have been the most convenient way for diagnosis and are widely used in the field. Typical symptoms of HLB of infected trees include blotchy mottle and/or variegated chlorosis of leaves, pale yellow leaves and stunting. The leaves become upright, followed by leaf drop from the laminar or petiole abscission zones, and at later stages extensive twig dieback occurs (Bové 2006). Often small-sized, lop-sided, and bitter tasting fruits with aborted seeds are found on HLB-infected trees. However, these symptoms seem not to be *Ca*. Liberibacter spp. specific, since a *Phytoplasma* sp. was reported to cause very similar symptoms in citrus to HLB in Brazil (Teixeira *et al*. 2008b). It is also reported that Las can survive years in citrus before showing obvious symptoms. HLB symptoms also vary with environment and trees become less symptomatic under high temperature during the summer in Florida.

To overcome the shortcomings of symptom-based diagnosis, various detection methods have been developed in recent years. DNA probes, conventional and Real Time PCR assays, electron microscope, enzyme-linked immunosorbent assays (ELISA) and biological indexing have been reported successful for diagnosis (Bové *et al.* 1993; Garnier and Bové 1993; Tian *et al.* 1996). Electron microscopy (EM) was the only reliable detection method for Liberibacters following the first EM observation of a "mycoplasma-like organism" in phloem tissues of HLB-infected citrus in 1970 (Laflèche and Bové 1970) until the first DNA probe specific to Liberibacters was designed in 1992 (Villechanoux *et al.* 1992). However, EM proved to be time-consuming and was unable to distinguish between the species of Liberibacter. A method based on the detection of a fluorescent gentiosyl-glucoside from infected fruits and bark was developed in 1968 (Schwarz 1968a) for confirmation of the disease. However, this method soon proved non-specific, since stressed trees also contained the same marker (Schwarz 1970). Biological indexing using plant indicators, such as sweet orange (Schwarz 1968b) and Ponkan mandarin (Matsumoto *et al.* 1968), was another confirmatory test of HLB. Monoclonal antibodies were raised for ELISA tests for the first time in 1987 (Garnier *et al.* 1987), but they were too isolate-specific to be used for general HLB detection. Two DNA probes specific to Las or Laf were later developed (Villechanoux *et al.* 1992), but the detection sensitivity of the dot-blot hybridization using these probes was similar to that of EM (Jagoueix *et al.* 1996). Recently, an iodine reaction (IR) kit was developed (Onuki *et al.* 2002) based on the elevated starch accumulation in HLB-diseased citrus leaves (Schneider 1968). However, the IR method is only a little better than the observation of visual symptoms of the disease. Diagnosis of HLB based on PCR methodology (Conventional PCR and Real Time PCR) has gained popularity when compared to other methods due to its sensitivity and reliability.

Conventional PCR

Since its first introduction by Kerry Mullis in 1983 (Mullis 1990) for which he won the Nobel Prize in 1993, PCR has become a powerful technique for the detection and identification of plant pathogens. The first set of 16S rDNA-based primers (OI1/OI2c and OA1/OI2c) was designed for conventional PCR to detect Las and Laf, yielding a fragment of 1160 bp in size (Jagoueix *et al.* 1996). A time-consuming enzyme digestion of the 1160 bp PCR product with *Xba*I was subsequently needed to distinguish between the two Liberibacter species. At the same time, one specific primer set was developed for detection of Las in China (Tian *et al.* 1996). In 1999, a second set of conventional PCR primers (A2/J5) were developed based on the β-operon ribosomal protein gene by Bové's group in France (Hocquellet *et al.* 1999). This set of primers allowed for the identification of the two Liberibacter species directly by separating the different sized amplicons generated by the primers (703 bp for Las and 669 bp for Laf). Also in 1999, another primer set was developed specific to Las based on DNA fragments obtained directly from HLB-infected citrus (Hung *et al*. 1999). This set of primers produces an amplicon of 226 bp and is specific only for strains of Las. Since none of the primer sets reacts with Lam (Teixiera *et al.* 2005), a new set of 16S rDNA-based specific primers (GB1/GB3) were developed, producing an amplicon of 1027 bp.

The universal 16S rDNA-based primer set fD1/rD1 (Weisburg *et al.* 1991) was used in the first round of the nested PCR assays to improve the detection sensitivity of conventional PCR assays with the 16S rDNA-based primer sets OI1/OI2c for Las (Deng *et al.* 2007) and GB1/GB3 for Lam (Teixeira *et al.* 2005). The primer set was also nested with another 16S rDNA-based primer set CGO3F/CGO5R for detection of Las in *Murraya paniculata* (Zhou *et al.* 2007). The loop-mediated isothermal amplification (LAMP) method was developed for detection of Liberibacters in laboratories without access to a thermal cycler (Okuda *et al.* 2005). Although the LAMP method is similar in sensitivity to conventional PCR assays, it is more vulnerable to contaminations than the later (Li *et al.* 2007).

Real-time PCR

Since Applied Biosystems (ABI) developed the first commercial real-time instrument in 1996 (Heid *et al.* 1996), real-time quantitative PCR (qPCR) has become the most accurate and sensitive method for the detection and quantification of nucleic acids yet devised (Shipley 2006). The first qPCR TaqMan probe Liberibacter detection was designed in 2004 (Liao *et al.* 2004) based on the 16S rDNA fragment amplified by the conventional PCR primer set OI1/OI2c (Jagoueix *et al.* 1996) from a HLB-infected citrus plant in Fujian, China. However, neither of the two primers BarF2 and Back2 is specific to Liberibacters. The amplicon produced by these two primers is 441 bp, which is out of the amplicon size range (50-250 bp) that is generally used for for real-time PCR (Wang and Seed 2006). This long amplicon resulted in decreased PCR efficiency and low detection sensitivity resulting in a detection limit of about 1,000 copies of template of cloned plasmid DNA per reaction.

In 2005, species-specific TaqMan probe-primer sets, HLBaspr, HLBafpr and HLBampr were developed for detection and identification of the three known species of Liberibacter in multiplex PCR with the positive internal control TaqMan probe-primer set COXfpr targeting the host plant cytochrome oxidase gene (Li *et al.* 2006a). The detection limits of the three HLB probe-primer sets are down to 1 to 10 copies of Liberibacter's 16S rDNA per reaction and their PCR efficiency is up to 99.90%. In addition, the detection limit and the PCR efficiency of the positive internal control probe-primer set COXfpr are almost the same as those of the HLB probe-primer sets, which allows for accurate estimation of the ratio of the Liberibacter DNA to the host plant DNA in total DNA extracts obtained from field samples. The HLBaspr set has been successfully applied in detection, identification and quantification of Las in host plants of citrus (Tatineni *et al.* 2008; Li *et al.* 2009) and in the psyllid vector (Manjunath *et al.* 2007). Recently, a very sensitive and stable positive internal control TaqMan probe-primer set WGfpr was successfully developed for real-time qPCR multiplexed with HLBaspr or HLBafpr or HLBampr for detection, identification and quantification of the three known species of Liberibacter in the psyllid vector (Li 2008).

Based on the β-operon protein gene of a DNA isolate obtained from HLB-infected citrus in Quangxi, China in 2006, a TaqMan probe-primer set CQULA04f/r/p10 was developed specifically for detection and quantification of Las. This probe and primer set has a similar PCR efficiency and sensitivity as the 16S rDNA-based HLBaspr (Li *et al.*

2006a). This primer pair has also been used in a SYBR Green real-time PCR (Wang *et al.* 2006). In 2008, another β-operon-based primer set was developed in Brazil for SYBR Green real-time PCR to study the distribution and quantification of Lam in citrus plants (Teixeira *et al.* 2008a). The PCR efficiency and sensitivity of this β-operon-based qPCR primer set are similar to those of the 16S rDNA-based TaqMan probe-primer set HLBaspr (Li *et al.* 2006a, 2007).

Recently a panel of 276 DNA extractions was sent to 13 different laboratories that are doing routine HLB diagnosis using a variety of methods and equipments. The methods used included conventional PCR with 16S rDNA primers and qPCR using 16S rDNA, β-operon primers, and unpublished putative DNA polymerase primers. Detection methodologies included ethidium bromide, TaqMan probes, and other fluorescent dye technologies. The samples included known HLB positive, known HLB negative, and a variety of field samples of unknown status. The results from the panel testing confirmed that under real-life testing conditions, the qPCR testing methodology was more sensitive than the conventional PCR. With the exception of labs that had specific reagent or equipment problems, all of the labs correctly identified the samples >93% of the time. However, most of the labs missed one or more of the positive samples and several of the labs had what were considered false positive results (i.e. positive results from the known negative samples). Thus, both false positive or false negative were observed among the labs. Within the labs using real time PCR systems and the same primers, there did not appear to be any difference between machines, reagents or detection systems. Similarly, there did not appear to be any differences in sensitivity for tests using primers based on different genome regions (16S, β-operon, or DNA polymerase nucleic acid sequences) (M. Irey *et al*. unpublished).

In summary, the current PCR methods have been very successful in the detection of the HLB bacterium. However, it should be noted that all the reported primers/probes are based on the very limited sequence data that were available at the time (16S rDNA, beta-operon, and DNA polymerase). False positive results have been reported (Tatineni *et al.* 2008; Teixeira *et al.* 2008a). It is probably due to the fact that all the sequences used are highly conserved. However, it is expected that primers/probes based on HLB specific genes will be available in the near future given the progress being made by several labs in HLB genome sequencing. The panel testing by the different labs and our previous work has indicated that there is always the possibility of false positive or false negative results using one single method. Thus, the use of a combination of different methods for final diagnosis is suggested (Tatineni *et al.* 2008).

IN PLANTA DISTRIBUTION AND SYMPTOMOLOGY

In planta distribution of *Ca.* L. asiaticus

Previous data indicated that the Las cells were unevenly distributed in different tissues of infected sweet orange trees (Tatineni *et al*. 2008). Las populations ranged from 14 to 137031 cells/µg total DNA in the various tissues, representing a 10,000-fold difference in bacterial population density. Uneven distribution has also been observed for Lam (Teixeira *et al.* 2008a). Las is found in most tissues of infected sweet orange trees including leaves, floral tissues (petals, pistils and stamens) and fruit tissues (peduncle, columella and seed coat). Even though no obvious symptoms were observed on floral parts, the HLB bacterium was detected in petals, pistils and stamens of flowers collected from infected trees. The HLB bacterium has been detected in small young fruit, and different fruit parts including peduncles, columella, and seed coat (**Fig. 1**). Seed transmission of the HLB bacterium has been suggested by Tirtawidjaja (1981). Further study is needed to address the seed transmission issue since the previous study on seed transmission was solely based on symptoms and was not confirmed with PCR and other means. Other bacteria, such as *Xylella fastidiosa* (Li *et al.* 2003), *Erwinia stewartii* (Khan *et al.* 1996), and Phytoplasma (Jiang *et al.* 2004), have been found to be seed-born even though the potential mechanisms involved are still unclear. Detection of the citrus HLB pathogen inside the fruit and seed may have important implications in preventing the spread of the citrus HLB disease. The distribution pattern of Las *in planta* was similar to the causal agent of mulberry dwarf phytoplasma, which was found in leaves, flowers, fruit, seed coats, and roots (Christensen *et al.* 2004). This might be due to the fact that both the HLB bacterium and Phytoplasma are phloem limited bacteria.

It has been reported that Lam population varied from 4.6×10^5 to 1.1×10^8/g, with an average value of 2.7×10^7/g in blotchy mottle sweet orange leaves (Lopes pers. comm.). Compared to Lam, preliminary data indicate that Las seems to reach a higher population level in the symptomatic leaves ranging from 4.37×10^7 to 3.38×10^8/g with an average of 1.94×10^8 (Wang, unpublished). This may be one reason why Las is more virulent than Lam (Lopes pers. comm.).

Symptomatic leaves seem to have a higher concentration of the Las bacterium than that of non-symptomatic leaves. This confirms observations made from earlier EM observations of symptomatic and asymptomatic midribs (Bové 2006). Higher Lam populations were also observed in symptomatic leaves in Brazil (Teixeira *et al.* 2008a). This might suggest that a threshold Las concentration is required for symptom development. It should be pointed out, however, that those population studies are based on genomic DNA copies *in planta*. It has been known that genomic DNA can be stable from several days to three weeks after cell death (Josephson *et al.* 1993; Masters *et al.* 1994). Consequently, quantitative analysis of bacterial populations based on total DNA can be overestimating the level of bacteria present (Nocker and Camper 2006). Efforts should be made to differentiate between dead and alive bacterial cells in the future.

The citrus HLB pathogen can move from the inoculation site to different parts of the plant. Las was detected in sink organs, such as young leaves, fruit, flowers, and roots from infected citrus trees in citrus groves, which is a good indication of its direction of movement within the phloem. With the only source of infection being a graft inoculation, greenhouse experiments clearly indicate that Las can move from the inoculation site to different parts of the tree, demonstrating the systemic movement of pathogen from the site of infection to different parts of the plant (Tatineni *et al*. 2008). Detection of Las inside the bark tissue, leaf midribs and roots from infected citrus trees clearly also suggest that the pathogen is transferred systemically through the continuous sieve tube system. The detection of the HLB bacterium in roots may explain why many symptomless trees pruned to the stump level developed HLB symptoms in new growth flushes (Lopes *et al.* 2007; Tatineni *et al*. 2008). The citrus HLB pathogen is about 2 µm long and 0.2 µm in diameter (Bové 2006). The pores on the sieve plates range from a fraction of a micron to about 14 µm (Easu and Cheadle 1959). Observation with EM indicated that *Ca.* Liberibacter spp. can move through the sieve plate pore in periwinkle, although no such observation has been made in citrus (Brlansky pers. comm.). It seems that the pathogen can move freely through the sieve pores along with the assimilate flow from leaves to sugar consuming plant organs, as has been observed with phytoplasma (Christensen *et al.* 2004; Jiang *et al.* 2004). However, it is not known whether the citrus HLB bacterium can pass through the pore-plasmodesmata unit between sieve elements and the companion cells, since they are much smaller (Christensen *et al.* 2004). Interestingly, phytoplasmas were documented in both companion cells and phloem parenchyma cells even though the mechanism of movement into phloem-related cells remains unknown (Siller *et al.* 1987; Li *et al.* 2007).

HOST RESPONSE TO HLB INFECTION

Microarray analysis of host response of sweet orange to HLB infection

The Affymetrix citrus microarray, which contains around 33,000 citrus probe sets from several citrus species and hybrids, was used by two groups including the senior author to characterize the host response of sweet orange to HLB infection under greenhouse conditions (Albrecht and Bowman 2008; Kim *et al.* 2009). It is unlikely that the entire *C. sinensis* transcriptome is represented on the array since the genome sequence of *C. sinensis* is still incomplete. Despite this, the array provides the most powerful tool to date for exploring global citrus gene expression. Both groups have found that HLB has tremendous effect on host gene expression. Interestingly, HLB seems to have more effect on the plant at later stages than the early stage of infection, since only 279 genes were differentially expressed 5-9 weeks after inoculation while 515 genes were differentially expressed 13-17 weeks after inoculation (cutoff log2 = ± 2 m; FDR ≤0.05) (Albrecht and Bowman 2008). In our study, the expression of a total of 624 genes was significantly changed: 307 genes were up-regulated and 317 genes down-regulated (cutoff log2 = ± 1.5; FDR ≤0.05) eight months after inoculation in the greenhouse. The host genes that showed changes in expression pattern induced by Las infection were related to plant pathogenesis/stress, anthocyanin biosynthesis, cell wall metabolism, cell division, detoxification, lipid metabolism, metabolite transport, metal transport, nucleotide metabolism, phenylpropanoid/flavonoid/terpenoid metabolism, phytohormones, protein kinase, protein metabolism, protein-protein interaction, signal transduction, sugar metabolism, transcription/translation factors and unknown/hypothetical genes. The broad range of host genes affected by Las infection suggests profound disturbances in plant metabolism. In the categories of cell cycle, cell wall metabolism, lipid metabolism, nucleotide metabolism and protein kinase, the majority of genes in a functional category were down-regulated in inoculated trees. In contrast, the majority of functional category genes associated with metal transport and signal transduction were mostly up-regulated (Kim *et al.* 2009). Approximately 5% of the genes with altered expression patterns were related to pathogenesis or stress. This suggests that Las survives in the phloem by disrupting the plant defense system. Alteration of host defenses, including the basal defense pathways, gene-for-gene resistance and non-host resistance, was shown to be critical for pathogenesis by *Pseudomonas syringae* (Nomura *et al.* 2005).

It was shown that genes associated with stress or abiotic and biotic stimulus were not induced greater than four-fold 5-9 weeks after inoculation (Albrecht and Bowman 2008). Only a few genes, such as the genes encoding a bacterial-induced peroxidase precursor, lipoxygenase and heat shock protein were induced greater than four-fold 13-17 weeks after inoculation (Albrecht and Bowman 2008). These findings suggest that the host could not effectively suppress the pathogen and resulted in the compatibility of HLB pathogen with sweet orange. A comparison of HLB-infected plant and healthy plants showed more changes in plant defense related genes. Over 10% of the genes significantly regulated in plants infected with Las 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 Las 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 the host response to citrus blight (Derrick and Timmer 2000). The up-regulation of PR genes in the host may be an indication of the activation of defense mechanisms that lead to processes, such as callose deposition in and around phloem tissues. Several defense-associated transcription factors that bind to promoter elements of individual defense-related genes were also up-regulated including WRKY4, WRKY6 (Eulgem 2000; Dong *et al.* 2003; Kalde 2003), ERF-1, ERF-2 (Gutterson and Reuber 2004), TGA (Jakoby *et al.* 2002; Krawczyk *et al.* 2002), and R2R3-MYB (Kranz *et al.* 1998; Stracke *et al.* 2001). Approximately half of the PR genes were down-regulated by Las infection. Numerous PR genes were also up- or down-regulated in both virulent and avirulent *Pseudomonas syringae* infections in *Arabidopsis* (Tao *et al.* 2003). It has been suggested that PR genes are up-regulated in a more intense and/or accelerated manner during the incompatible interaction (Maleck *et al.* 2000). Suppression of host defenses, including basal defense, gene-for-gene resistance and non-host resistance was shown to be critical for pathogenesis (Nomura *et al.* 2005). Both the transcriptional profiles of sweet orange to HLB infection at early or later stages indicated that Las can escape or defeat the plant defense system and survive in the phloem (Albrecht and Bowman 2008; Kim *et al.* 2009).

Starch has been shown to accumulate in leaves of HLB-infected plants (Schneider 1968; Bové 2006). Over 4% of the genes with significantly altered regulation in Las-inoculated plants were 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 (Zeeman *et al.* 2007). 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 (Nakamura *et al.* 1995). 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, AGPase, starch synthase and granule-bound starch synthase were up-regulated in HLB infected citrus leaves. The induction of AGPase was also observed (Albrecht and Bowman 2008). Interestingly, genes directly associated with photosynthesis were not influenced by HLB infection. The up-regulation of key starch biosynthetic genes in photosynthesis, together with restricted movement of photosynthates from leaves due to phloem plugging, is postulated to lead to the accumulation of starch in HLB infected leaves (**Fig. 1**) (Kim *et al.* 2009).

Phloem plugging has been demonstrated in HLB-infected plants (Schneider 1968; Bové 2006; Kim *et al*. 2009). Mechanisms of sieve pore plugging can involve phloem protein 1 (PP1), PP2 and callose (Knoblauch and van Bel 1998; Dinant *et al.* 2003). PP2, a dimeric poly-GlcNAc-binding lectin, covalently cross-links with PP1 via disulphide bonds, forming polymers that closed sieve pores (Read and Northcote 1983). This response is normally accompanied by the synthesis of the β-1,3-glucan callose (McNairn and Currier 1968). The *pp2* gene was highly up-regulated in HLB-infected leaves, and the protein product likely contributed to phloem blockage (together with callose deposition) observed in micrographs (**Fig. 1**). Additionally, PP2 has been suggested to interact with a variety of putative signaling RNAs (Gomez and Pallas 2001; Owens *et al.* 2001) and as such may interfere with communication between source and sink organs and nutrient transport. A similar result was reported on the induction of *pp2* gene (Albrecht and Bowman 2008).

HLB infection results in phloem damage, plugging of sieve pores and interference with sucrose transport

Schneider has done a very comprehensive study of the anatomical changes that result from greening infection with Laf. In his study, the midribs of uninoculated and HLB-infected

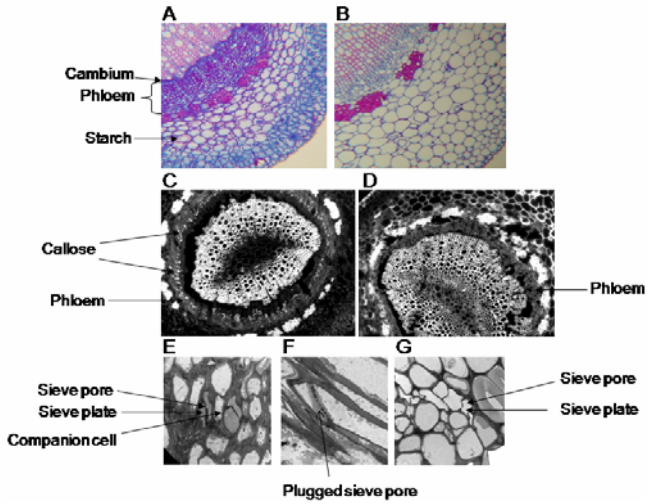

Fig. 1 Anatomical analysis of midrib phloem tissues of Huanglongbing (HLB) pathogen infected and healthy sweet orange (*Citrus sinensis*). Light micrograph (LM) of methylene blue-azure A and basic fuschin staining (**A** and **B**): staining shows thickened and disrupted cell walls of phloem tissues of affected citrus (**A**) compared to the healthy control (**B**). Starch particles stained red (**A**) are also observed in the mesophyll parenchyma cells of the HLB infected plant only. The callose staining of citrus midrib in 0.05% aniline blue solution observed under a fluorescent microscope with UV filter (**C** and **D**). Light areas reveal callose in the phloem. The infected midrib is full of callose in the phloem tissue (**C**) while the healthy control does not show light staining of callose (**D**). Transmission electron microscopy (TEM) of phloem tissue (**E**, **F**, and **G**). Cell walls are thicker and disrupted and sieve pores are plugged in the HLB infected plant (**E** and **F**) while not in the healthy plant (**G**).

sweet orange leaves were stained using the general polychromatic/complex carbohydrate staining procedure with methylene blue-azure A and Basic Fuschin (Schneider 1981) and observed with light microscopy. Recently, a detailed study was conducted to compare the process of leaf chlorosis and phloem collapse due to different causes using a similar methodology (Albrigo unpublished). HLB affected leaves were compared to nutrient deficient, chlorotic and healthy leaves using light and transmission electron microscopy. The major symptoms in leaves indicative of HLB disease were asymmetrical chlorosis, starch accumulation and phloem necrosis. Large amounts of starch accumulated in the mesophyll, epidermal, oil glands and vascular bundle parenchyma in leaves from the HLB pathogen infected trees (**Fig. 1A** and **1B**). This is consistent with the up-regulation of three major starch synthesis genes. Starch accumulation occurred first in the spongy mesophyll and then in the palisade layer. The outer membranes of the chloroplasts were always retained, with no evidence of rupturing, even with large accumulations of internal starch. As starch accumulated, the internal membranes of the plastids degenerated. Sieve plates in necrotic phloem were found to be totally occluded by an amorphous plugging material, similar to callose (**Fig. 1C** and **1D**) and a filamentous plugging material, which may contain significant amounts of P-protein-like material, occluded whole sieve elements. Phloem collapse and necrosis were observed in the petiole, mid-vein and minor vein branches in HLB affected leaves (**Fig. 1E-G**). Phloem necrosis was also observed in asymptomatic leaves before starch accumulation and chlorosis. In contrast to HLB, necrosis of the phloem in Zn and Fe deficient leaves was found to occur after the development of chlorosis.

TEM analysis has proven to be very useful in the study the HLB bacteria in phloem. In all midrib samples observed by TEM, when present, Las existed as single cells and did not form visible aggregates in the phloem. Furthermore, no plug composed of Las cells was observed in phloem sieve pores. Given the size of a Las cell, it is unlikely that a single bacterium could plug a sieve pore since a bacterium is approximately 2 µm long and 0.1-0.2 µm in diameter (Laflèche and Bové 1970) while sieve plates pores range from less than 1 µm to about 14 µm (Easu and Cheadle 1959). Consequently, it is unlikely that the Las physically caused phloem blockage because multiple bacterial cells were not aggregated. Probably, the blockage is caused by the host response in the phloem that results in sieve pore plugging.

Las causes similar symptoms and host response in citrus as *Phytoplasma* spp., even though Las is Gram negative while *Phytoplasma* spp. are mollicutes and lack cell walls. In Brazil, it was reported that a phytoplasma closely related to the Pigeon pea witches's broom phytoplasma (16Sr IX) can cause similar symptoms as HLB on sweet orange (Teixeira 2008b). This is probably due to the fact that both pathogens colonize phloem sieve tubes and disrupt host mechanisms which eventually disrupt phloem transportation and therefore cause similar disease symptoms.

CONCLUDING REMARKS

Ninety years have passed since citrus HLB was first reported in southern China (Bové 2006). Citrus HLB has spread to most citrus producing areas of the world. The major impediment to citrus HLB research has been the difficulty of *in vitro* cultivation of the HLB organism, which is fundamental to understanding microbial physiology, morphology, genetics, and its virulence mechanism. Recently, Las was cultured *in vitro* (Davis *et al*. 2008). It was co-cultured with other bacteria closely related to *Propionibacterium acnes*. Separation of the two organisms in culture into two monocultures has not yet been achieved and Koch's postulates have not been completed. Further effort is needed to culture Las *in vitro*. HLB genomic DNA from the co-culture should help with the current genome sequencing. When obtained, the genome sequence of Las will help elucidate the biological characteristics of this citrus pathogen and the mechanisms of pathogenicity and insect-transmissibility. Hopefully, it will help in the development of effective ways of managing this devastating disease of citrus. Culturing and genome sequencing of the HLB bacterium are the two critical stepping stones for further study of citrus HLB disease.

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