PLANT DISEASES ASSOCIATED WITH '*Candidatus* LIBERIBACTER' SPECIES: CITRUS HUANLONGBING AND POTATO ZEBRA CHIP

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Abstract

'*Candidatus* Liberibacter' species are phloem-limited prokaryotes that are associated with citrus huanglongbing (HLB), zebra chip (ZC) disease, diseases of other solanaceous crops, and carrot psyllid damage. Citrus HLB (also referred to as greening and yellow shoot disease), potato ZC and diseases of other solanaceous crops are unique and possibly recently emerged host-pathogen(s)-insect vector pathosystems. HLB is distributed worldwide, while ZC occurs in North and Central America and in New Zealand. Several phloem-restricted bacteria are associated with citrus HLB and potato zebra chip (ZC) disorder or disease. These include '*Candidatus* Liberibacter' species '*Ca*. L. asiaticus' (CLas), '*Ca*. L. africanus' (CLaf), '*Ca*. L. americanus' (CLam) and '*Ca*. L. solanacearum' (CLso; syn. psylllaurous), as well as and phytoplasmas. HLB- and ZC-associated liberibacters, as well as phytoplasmas, are transmitted by psyllids. The involvement of '*Ca*. Liberibacter' species, and possibly phytoplasma(s), in the etiologies of HLB and ZC is suggested based on associative evidence; however, Koch's postulates *sensu stricto* have not been conclusively fulfilled for either disease. The potential role of other prokaryotes, viruses and possibly other agents in the etiologies of HLB and ZC are also unknown at this time. Management of Liberibacter-associated diseases is based on production and use of pathogen-free or healthy plant stock, quarantine measures regulating propagation and movement of planting stock, removal of affected plants, and reduction of psyllid populations with chemical insecticides. The most effective long-term management of Liberibacter-associated diseases is likely to be based on host resistance.

Introduction

Important vascular diseases of citrus and potatoes are caused by, or associated with, prokaryotes. These include bacteria that are characterized by distinct tissue specificities (i.e., phloem- or xylem-limited), distinct modes of pathogenesis and different modes of transmission. Among these are several '*Candidatus* Liberibacter' species.

'*Ca.* Liberibacter' species are closely related members of the α-*Proteobacteria* sub-division of prokaryotes. They are Gram negative bacteria surrounded by a triple-layered cell envelope consisting of an outer cell wall, peptidoglycan layer and inner cytoplasmic membrane. They are fastidious, unculturable, phloem-limited prokaryotes and are associated with citrus huanglongbing (HLB), zebra chip (ZC) disease and diseases of other solanaceous crops, and recently associated with carrot psyllid damage (5,8,19,20,32,38,39,44,55). Citrus HLB (also referred to as greening and yellow shoot disease) and potato zebra chip (ZC), as well as other diseases of solanaceous crops, are destructive diseases that cause significant economic losses during crop production and processing. HLB is widespread in most citrus-producing worldwide (5,20). ZC affects potatoes in North and Central America, the Caribbean and in New Zealand (8,32,33,44,55). Other solanaceous crops are also affected in New Zealand (32).

Three species of HLB-associated bacteria include '*Ca.* Liberibacter *asiaticus*' (CLas), '*Ca*. L. *americanus*' (CLam) and '*Ca*. L. *africanus*' (CLaf). CLas is the virulent and most widely distributed HLB-associated bacterium globally (5,17,19,20,52). CLam and CLaf occur in Brazil and Africa, respectively (5,19). Lam has also been reported to occur in China (5); however, this has not been confirmed. '*Ca.* L. *solanacearum*' (CLso) is associated with ZC, psyllid yellows and haywire diseases of potato (8,32,44,55), as well as with diseases of other solanaceous crops (8,9,33,44,55). '*Ca.* Liberibacter psyllaurous' (CLps) was reported to be associated with "psyllid yellows" of tomato and potato (21). However, CLso and CLpsy are probably synonyms of the same Liberibacter species. CLpsy was not described and validly published, and no reference material is available, while CLso was validly published and reference material is available (21,32,44,55). Accordingly, it is suggested that '*Ca*. Liberibacter solanacearum' (21) be the preferred species name. CLso currently occurs in North and Central America, New Zealand, and Europe (Finland) (31) and possibly elsewhere (Chen, et al*, unpublished data*). HLB-associated Liberibacters are transmitted by *Diaphorina citri* (CLas, CLam) and *Trioza erytreae* (5,19), while CLso is transmitted by *Bactericera cockerelli* (8,21,39,44,55). The CLso strain or variant reported in carrots in Europe is associated with, and presumably transmitted by, the psyllid *Trioza apicalis* (38). A subspecies of CLaf, '*Ca*. Liberibacter africanus subsp. capensis' (CLafc), is associated with Cape chestnut in South Africa (13).

The genomes of a Florida strain of CLas and U.S. strain of CLso have been sequenced (13,14). The size of these genomes is approx. 1.23-1.26 Mb. Briefly, there are 1,136 and 1,126 protein coding regions in the CLas-Florida strain and CLso-U.S. strain genomes, respectively. These genomes reflect reduced or complete absence of some metabolic enzymes and secretion systems in these genomes. There are 867 conserved proteins, of which 531 proteins share ≥70% similarity. These may represent the core genome. The remaining 336 proteins are more diverse and a significant portion of these are associated with membrane and transport functions. These may help define Liberibacter speciation. These CLas and CLso genomes differ quantitatively by ≥25% among six functional categories of clusters of orthologous groups (COG). Five of the COG are related to external interactions associated with a pathogenic lifestyle. Sequencing of the genomes of a Chinese strain of CLas, CLam and CLaf is in progress.

At least two phytoplasmas have also been associated with HLB symptoms in Brazil - the pigeon pea witches' broom phytoplasma '*Ca*. Phytoplasma phoenicium' (16Sr IX group) (5,48), and in China – the aster yellows phytoplasma '*Ca*. Phytoplasma asteris' (16Sr I group) (6). The phloem-limited prokaryote, *Spiroplasma citri*, causal agent of citrus stubborn disease, is phloem-limited and causes some HLB or HLB-like symptoms; however, *S. citri* has not been specifically associated with HLB. Nor has the phloem-limited '*Ca*. Phytoplasma aurantifolia', associated with citrus witches-broom, been linked to HLB.

The association of other bacteria-like organisms (BLOs) with HLB and ZC is not clear. However, PCR primers that amplify DNA from the cucurbit yellow vine disease-associated, phloem-limited BLO, referred to as an atypical strains of *Serratia marcescens*, specifically amplified a 690 bp product in extracts of ZC-affected potato tubers (56). Although identical sequences were not identified, the sequence of this amplicon had a similarity >97% to various enteric bacteria from diverse plant and insect sources (56).

Symptomatology

The symptomatology of HLB and ZC have been extensively described (5,8,18,19,20,54).

Symptoms of HLB include blotchy leaf mottle; pale yellow leaves; small, erect leaves; small, green, circular spots on leaves; leaf and fruit drop; twig dieback; yellow shoots; and overall tree stunting and death. Leaf veins may be enlarged and corky (5,18,19,20). Fruit are small, lopsided and bitter-tasting with discolored and aborted seeds, and discolored vascular bundles at the peduncular end of the columella (5,18,19,20). A color inversion of the fruit occurs when the stylar end remains green while the stem end turns yellow or orange (5,18,19,20). Nutrient deficiency-like symptoms may also occur on HLB-affected plants (5,18,19).

Foliar symptoms of ZC resemble those of phytoplama-associated diseases of potato, and include chlorosis, leaf scorching, swollen nodes, upwardly curled leaflets, early senescence, aerial tubers, and early plant decline (8,39,44,55). Subterranean symptoms include collapsed stolons, enlarged lenticels, brown discoloration of the vascular ring and necrotic flecking of tuber tissue (8,38,43,54). Tuber slices fried for potato chips exhibit characteristic dark blotches and stripes or streaks that coincide with the medullary ray tissues of the tuber (8,39,44,55).

Disease Diagnosis

Diagnosis of HLB and ZC in the field based on symptoms can be difficult as symptoms may not necessarily be HLB- or ZC-specific. Development and appearance of HLB and ZC symptoms can be affected by environmental conditions and host genotype, as well as plant age, horticultural and agronomic health of plants, and other factors (5,19,20,54). In addition, Liberibacter-infected citrus trees may not exhibit symptoms for several years (5,19,54). Nevertheless, more or less typical symptoms of HLB- and ZC-affected plants are convenient and are widely used for detection and diagnosis of these diseases (5,19,54).

Liberibacter Detection and Identification

A variety other disease diagnostic methods have been developed to supplement or confirm field-based diagnosis of HLB and ZC (2,4,21,23-26,28-31,35,36,54). However, PCR-based methods to detect and identify HLB- and ZC-associated Liberibacters are currently the most widely used methods. Conventional PCR primers are generally based on 16S rRNA and the β-operon ribosomal protein gene (4, 21,23-26,28-31,35,36,54). The 16S rRNA primers have also been nested to improve the detection sensitivity of conventional PCR (4,11,12,41). Loop-mediated isothermal amplification (LAMP), developed for CLas detection, can be used when a thermal cycler is not available (41).

Real-time quantitative PCR is generally the most reliable, sensitive method for detection and quantification of Liberibacter DNA sequences in plant and insect tissues (28,29,41). These have generally been based on amplification of 16S rRNA, β-operon and putative DNA polymerase sequences using various primers and primer/probe combinations with TaqMan or SYBR Green (41). Species-specific TaqMan probe-primer sets have been developed for CLas, CLaf and CLam in multiplex PCR with the positive internal control TaqMan probe-primer set COX targeting the host plant cytochrome oxidase gene (41). Recently, a Liberibacter PCR-based detection system using a pair of universal primers designed in flanking regions shared among CLas, CLaf, CLam and CLso, with polymorphisms in the amplicon regions representing unique sequence characteristics for each species, distinguishing these species by high resolution melting curve analyses (Lin et al, *unpublished*). In contrast to multiplex PCR or use of multiple primer sets or primer/probe combinations commonly used for detecting different Liberibacter species, this method uses only one pair of primers.

The current PCR-based methods to detect and identify HLB- and ZC-associated Liberibacters have generally been successful. However, the primers and primer/probe combinations currently used are based on very limited available genomic sequences that are highly conserved (41). The complete sequence of the genome of a Florida Las strain has been reported (14). Sequencing of the sequences of the genomes of other Las strains, Laf, Lam and Lso are in progress (Lin et al, *unpublished*). Identification of Liberibacter-specific genes through comparative analyses of Liberibacter genomes should lead to improved HLB detection and diagnosis, and Liberibacter detection and identification. In addition, identification of HLB- and ZC-specific biomarkers and genetic signatures that clearly signal early stages of HLB- and ZC-development in clinically asymptomatic trees should lead to development of enhanced disease detection and diagnosis. Finally, novel platforms (e.g., microarrays, lateral flow cytometry, electronic noses) and technology (e.g., ground-based spectroscopic- and imaging-methods) are being developed for field-deployable disease detection systems.

Liberibacter Diversity

The genetic diversity, primarily at several loci in the *rrs* and *rpl* genes, and in the *omp* locus, of HLB- and ZC-associated Liberibacters is well-documented (4,6,17,24-26,32,53). While these loci are useful for Liberibacter detection and determination of phylogenetic relationships, they are highly conserved and reveal little strain diversity. Single-nucleotide polymorphisms in the *omp* locus were used to group CLas strains from Thailand and Nepal, but not from the Philippines and China-Behai (6,10). Bacteriophage-type DNA polymerase were used to identify three clusters in the Southeast Asian CLas population and one cluster of Indonesian CLas strains, while other CLas clusters were not correlated with geographical distribution (6,50). Analyses of a genetic locus in the CLas genome containing tandem repeats in the bacteriophage repressor protein C1 gene showed that the CLas in Guangdong (People's Republic of China) was significantly different from that in Florida (United States (7). Moreover, two tandem repeat number sub-groups, one widely distributed in Florida and the other limited to Central Florida, were identified (7).

Fifty-eight CLso strains detected in potato in North America were grouped into two genetic clades based on analyses of sequences in their 16S rRNA and partial 16S-intergenic spacer-23S rRNA regions (55). However, the CLso strains from ZC-affected potatoes could not be effectively distinguished from CLso strains from "psyllid yellows"-affected potatoes using existing PCR protocols (55). The relationship between distinct diseases, such as ZC and "psyllid yellows" of potato, and the same bacterium is not clear. Comparative genetic analyses of CLso populations in the United States, New Zealand and Europe have not been made.

Disease Epidemiology

HLB and ZC are unique, complex pathosystems. The etiologies of HLB and ZC have not been conclusively established. '*Ca*. Liberibacter' species are most frequently, but not the only potential pathogen(s), associated with these diseases. Koch's postulates *sensu stricto* have not yet been fulfilled for any of the Liberibacters associated with HLB (CLas, CLam, CLaf) and ZC (CLso). However, the incidence of both HLB and ZC is closely associated with the presence of psyllids. All of the HLB- and ZC-associated Liberibacters occur in, and are transmitted by, *D. citri* (CLas, CLam), *T. eryetreae* (CLaf) and *B. cockerelli* (CLso) (5,8,19,36) while the carrot yellows-associated CLso strain or variant is presumed to be transmitted by the carrot psyllid (*T. apicalis*) (39).

Citrus trees with typical HLB symptoms are occasionally PCR negative for Liberibacter (6,48). Although Liberibacters are the prokaryotes most frequently associated with HLB and ZC, they are not uniformly distributed and are generally present in relatively low levels in diseased plants (20,47,51). In addition, CLas may not always be associated with citrus trees exhibiting characteristic HLB symptoms (5,41).

Various levels of CLas and CLso have reported to occur in tissues from different parts of HLB- and ZC-affected citrus trees by qPCR. These include root, leaf midrib, petal, pistil, stamen, peduncle, columella, seed coat, young whole fruit and bark, but not in endosperm, from HLB-affected citrus (46), and midribs, petioles, leaf blade, whole stalk, stalk epidermis, stalk cortices, aerial tubers, root epidermis, root cortices and tubers from ZC-affected potatoes. The highest level of CLas occurred in peduncle tissue, although a relatively high level of CLas was detected in root tissue (47). The highest levels of CLso occurred in root tissues (epidermis, cortices) and tubers (28). However, it is not clear if seed-transmission of HLB-associated Liberibacters is epidemiologically significant. CLso has not been reported to occur in true potato seed. Generally, seed pieces from tubers from ZC-affected plants germinate poorly or not at all;

however, transmission of CLso via seed potatoes resulting in ZC in the next generation is not clear.

CLas and CLso have been detected in a broad range of plants (5,11,12,19,54,55) on which the psyllid vectors occur. These alternate hosts could be sources of inoculum to initiate new infections at the beginning of the season, or during the growing season when appropriate tissue is available for psyllid feeding. However, the role of LIberibacter-containing alternate hosts in the epidemiologies of HLB and ZC is not clear. HLB- and ZC-associated Liberibacters are detectable, and may be endosympbionts, in their psyllid vectors (20,21).

The HLB- and ZC-associated Liberibacters are closely related genetically (32,34,44,55). However, CLso is not been detected in naturally-occurring HLB and has not been reported to occur in the ACP. Similarly, none of the HLB-associated Liberibacters has been associated with ZC and have not been detected in potato psyllids.

Two '*Candidatus* Phytoplasma' species, pigeon pea witches' broom (group 16Sr IX) and aster yellows (group 16Sr I), were detected in trees with characteristic HLB leaf and fruit symptoms in Brazil and China, respectively (6,48). In Brazil, none of the three HLB-associated Liberibacter species was detected in trees with HLB symptoms. In China, CLas, phytoplasma, or both CLas and phytoplasma were detected in trees with typical HLB symptoms . Psyllids can also transmit the two phytoplasmas that have been associated with HLB (5). However, vector(s), such as leafhoppers, planthoppers, and/or psyllids that transmit the pigeon pea witches' broom and aster yellows phytoplasmas from citrus to citrus have not been identified (5). The possible involvement of these phytoplasmas in HLB is unknown. Similarly, phytoplasmas have also been detected in ZC-affected potatoes, but have not been consistently associated with the disease.

The nature of the associations of several BLOs (e.g., Liberibacters, phytoplasmas) to HLB and ZC are not conclusively understood as Koch's postulates *sensu stricto* have not been fulfilled. All of these organisms colonize phloem tissue, disrupt host metabolism, and affect phloem transportation of carbohydrates resulting in disease or disease-like symptoms that may be similar to other diseases, nutritional problems or other stress factors (5,18,19,47).

In Planta Microbial Diversity-Disease Relationships

The genomes of the CLas and CLso small and bioinformatic analyses suggest that they do not have all of the genes encoding all the proteins necessary for housekeeping activities and metabolic pathways (14,20; Lin et al, *unpublished data*). This may indicate that Liberibacters are not able to cause disease independently, but may depend upon other endophytic microflora to provide missing metabolites to elicit disease (20). Qualitative and quantitative differences in the diversity of bacterial communities in citrus and potato have been reported (16,42,43,45,46,49-51). The endophytic prokaryote communities specifically associated with HLB have been evaluated (35,43,44), while the qualitative and quantitative diversity of bacterial endophytes associated with ZC is being determined. Little is known about the effects of HLB and ZC disease on the structure of microbial communities *in planta*, or the potential involvement of microbial endophytes on development of HLB and ZC.

Unique phylotypes and genotypes of bacteria have been associated with HLB-affected, but apparently not unaffected, plants (50,51). The majority of bacterial types in un-affected trees were similar to known plant growth-promoting bacteria, including *Bacillus, Burkholderia, Caulobacter, Lysobacter, Paenibacillus, Pantoea, Pseudomonas, and Stenotrophomonas* (51,52). *In planta* levels of most of these types of bacteria were reduced, while bacteria such as *Methylobacterium* and *Sphingobacterium* which were not detected in root samples from un-affected trees were present in root samples from HLB-affected trees (51). Representatives of the phylum *Actinobacteria*, particularly *Curtobacterium* species, were detected only in root samples from trees not affected by HLB (51). The endophyte *Curtobacterium flaccumfaciens* has been isolated frequently from citrus variegated chlorosis (CVC)-affected, but not asymptomatic, sweet orange trees and has been shown to reduce the severity of disease symptoms in periwinkle plants infected with *X. fastidiosa*-CVC pathotype (3). However, *X. fastidiosa* subspecies are limited to the xylem *in planta*.

Potatoes also contain a broad range of bacteria, including members of the α, β and γ *Proteobacteria*, Gram-positive organisms, and members of the (16,41,44,48). At least some of these are antagonistic to Gram-negative plant pathogenic bacteria of potatoes (41,48).

The potential role of bacterial or other microbial endophytes in HLB and/or ZC in disease development and/or symptom severity is unknown. However, microbial endophytes could provide components necessary for Liberibacters to colonize and elicit disease in citrus and potato. Alternatively, bacteria may exclude the HLB and ZC etiological agents by colonizing the same etiological niches, activate innate host plant resistance or elicit induced systemic resistance, or produce anti-microbial compounds or molecules.

Host Responses

HLB- and ZC-associated Liberibacters colonize the phloem of affected plants (5,19,22,32,33, 44,55). However, the distribution of these bacteria in different tissues of diseased plants is not uniform (22,31,46). The bacterial cells in TEM images of phloem of affected plants tend to occur as single cells rather than in aggregates (27,46) A wide range of phenotypic and genotypic responses associated with symptoms of HLB- and ZC-affected plants have been described (1,13,27,37,47). Starch accumulation in the leaf mesophyll and epidermal cells, oil glands and vascular bundle parenchyma and phloem collapse necrosis, along with degeneration of the internal membranes of plastids were associated with HLB symptoms (47). In addition, sieve plates in necrotic phloem became totally occluded by amorphous plugging material similar to callose and a filamentous plugging material (27,46). Layers of dead cells surrounding numerous, small, irregularly-shaped lesions throughout the parenchymatic medullary region, and vascular ring and cortex were associated with ZC symptoms in tubers (37). The internal discoloration in tubers from ZC-affected plants has been attributed to elevated levels of reducing sugars and phenolics and to general tissue necrosis (37,40). Fewer starch granules occurred in tuber tissue from ZC-affected plants that in tuber tissue from ZC-free plants (37).

Genes are differentially up- and down-regulated in HLB- and ZC-affected plants. Host genes whose expression was significantly altered in HLB-affected plants included those associated with plant pathogenesis/stress, anthocyanin biosynthesis, cell wall metabolism, cell division, detoxification, lipid metabolism, metabolite transport, metal transports, nucleotide metabolism, phenylpropanoid/flavonoid/terpenoid metabolism, phytohormones, plant defense, protein kinase, protein metabolism, protein-protein interaction, signal transduction, sugar metabolism, transcription/translation factors and unknown/hypothetical genes (27).

Disease Management

Effective management strategies for Liberibacter-associated diseases require early, rapid, sensitive, specific and reliable disease detection diagnostic methods. Currently, HLB and ZC management is based on avoidance, removal of inoculum sources, and reducing Liberibacter transmission. These include exclusion of the causal agents to prevent their introduction, establishment and spread via quarantines and certification of Liberibacter-free propagating material; eradication, if possible, before widespread and establishment of the disease-associated-Liberibacters; removal of inoculum sources by eliminating diseased plants and infected, but asymptomatic, plants if detected; geographical and/or physical isolation of plant stock and crop production systems; reducing ACP and potato psyllid populations with chemical insecticides (or via biocontrol agents if feasible) to reduce transmission of Liberibacters (). While biological control of the ACP may be an integral component of integrated pest management systems to manage HLB, biological control of the potato psyllid may have a limited role in the management of ZC. Other cultural practices to preclude or reduce psyllid spread and transmission of Liberibacters may also be effective in managing HLB and ZC. It is likely that the most effective management of HLB and ZC will be based on genetically or induced resistant hosts.

The most effective, sustainable management of Liberibacter-associated diseases is likely to be based on host resistance achieved through induction of innate host resistance (e.g., systemic acquired resistance), and by conventional breeding and/or transgenic improvement (15). However, available sources of tolerance to citrus HLB and potato ZC are limited at this time (15). Although existing citrus and potato genotypes exhibit varying levels of to HLB and ZC, respectively, there is no known genetic resistance to either disease.

Summary

- Citrus HLB & potato ZC are complex pathosystems with complex syndromes.
- Etiologies of citrus HLB & potato ZC have not been conclusively determined or demonstrated.
- Liberibacters and phytoplasmas have been associated with citrus HLB and potato ZC; however, the association of phytoplasmas with HLB is limited.
- Other potential pathogens may be involved in HLB and ZC etiologies.
- Liberibacters are genetically & pathogenically diverse
- There is a need for improved, robust, reliable disease detection methods and technology to detect clinically-asymptomatic plants and to detect and identify disease-associated Liberibacters with increased sensitivity and specificity to minimize false negatives and positive, and capable of high throughput processing and analyses, and are field-deployable.
- Liberibacter-associated diseases occur worldwide
- The biology, ecology and epidemiology of HLB- & ZC-associated Liberibacters need to be better understood.
- Effective, economical management of ZC likely to be based on integrated strategies involving host resistance to the pathogen(s) and psyllid vectors; inoculum exclusion, removal or reduction; vector control; cultural practices; and new novel approaches.

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