Huanglongbing: An overview of a complex pathosystem ravaging the world’s citrus

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Abstract  Citrus huanglongbing (HLB) has become a major disease and limiting factor of production in citrus areas that have become infected. The destruction to the affected citrus industries has resulted in a tremendous increase to support research that in return has resulted in significant information on both applied and basic knowledge concerning this important disease to the global citrus industry. Recent research indicates the relationship between citrus and the causal agent of HLB is shaped by multiple elements, in which host defense responses may also play an important role. This review is intended to provide an overview of the importance of HLB to a wider audience of plant biologists. Recent advances on host-pathogen interactions, population genetics and vectoring of the causal agent are discussed.

Keywords: Citrus greening; Diaphorina citri; host response; Huanglongbing (HLB); psyllid vectors


INTRODUCTION

Citrus is susceptible to a wide range of diseases caused by fungi, oomycetes, bacteria, nematodes, viruses and viroids (Timmer et al. 2000), but the most serious of these on a worldwide scale is now generally considered to be Huanglongbing (HLB), also known as citrus greening and in China as yellow shoot disease (Figure 1) (Bové 2006). HLB is one of the most complex diseases of citrus, with interactions among the pathogen, vector, hosts and the environment in its broadest definition (weather, soils, plant nutrition, presence of other pathogens and pests, etc.). Moreover, this is also coupled with the long latent period, inability thus far to culture the causal organism, and the lack of any known sources of natural resistance, making it a major challenge for researchers, regulatory agencies and the citrus industry.

HLB is associated with three species of the Candidatus Liberibacter genus: Candidatus Liberibacter asiaticus (Las), Candidatus Liberibacter africanus (Laf) and Candidatus Liberibacter americanus (Lam) (Bové 2006). Four additional subspecies of Laf have also been recognized: Candidatus Liberibacter africanus subsp. zanthoxyli (LafZ) and Candidatus Liberibacter africanus subsp. capensis (LafC), Candidatus Liberibacter africanus subsp. clausenae (LafCl), Candidatus Liberibacter africanus subsp. zanthoxyli (LafZ) and Candidatus Liberibacter africanus subsp. capensis (LafC) (Roberts et al. 2015) (Figure 2). All Ca. Liberibacter spp. belong to the Gram-negative alpha (α)-proteobacteria in the family Rhizobiaceae and are transmitted by two species of citrus psyllids, Diaphorina citri Kuwayama (Asian citrus psyllid: ACP) and Triozia erytreae (del Guercio) (African citrus psyllid) (Bové 2006). At least six additional species of psyllids colonize citrus and its close relatives (Halbert and Manjunath 2004). Of these, two species in addition to ACP and T. erytreae-are implicated in HLB transmission. Las has also been found infecting Cacopsylla citrisuga (Yang and Li 1984; Cen et al. 2012) and Diaphorina communis Mather (Donovan et al. 2012) but no actual transmission tests have been reported.

Theories about the origins of the disease are controversial, and it is unlikely that they will be conclusively proven. The Asian (Las) and African (Laf) forms most likely infected citrus in the respective continents through indigenous psyllid species transferring the causal bacteria from indigenous rutaceous plants to cultivated citrus. Descriptions of die-back of citrus in India in the 18th century (Capoor 1963) and the observations of farmers in southern China in the late 1800s...
(Zhao 1981) suggest that this disease has impacted citrus for over 100 years. The disease in Africa was noticed first in the late 1920s in areas where citriculture was expanding (Van der Merwe and Andersen 1937). Just over a decade ago, HLB was confirmed in the Americas, originally in São Paulo State in Brazil in 2004 (Teixeira et al. 2005a) and the State of Florida, USA in 2005 (Halbert 2005). The disease spread rapidly in both São Paulo and Florida, causing significant economic losses as it has in Asia for many years. HLB has moved into neighboring states in Brazil as well as Argentina and Paraguay (Lopes et al. 2013). In the USA, HLB has been detected in two other significant citrus producing states, Texas (Kunta et al. 2012; da Graça et al. 2015) and California (Kumagai et al. 2013) as well as in South Carolina, Georgia and Louisiana (Halbert et al. 2010). The disease is also widespread in several Caribbean countries such as Cuba (Luis et al. 2009), Jamaica (Oberheim et al. 2011), Belize (Manjunath et al. 2010) and in Mexico (Trujillo-Arriga et al. 2010). Other major citrus growing areas of the Mediterranean Basin and Australia are now under threat. HLB has moved west from Pakistan into Iran (Faghihi et al. 2009), threatening Turkey and beyond, and the African psyllid recently was found in Spain.

Figure 1. Huanglongbing (HLB) symptomatology. *Candidatus Liberibacter* sp. are interfering with various citrus physiological pathways and anatomical structures

As a result HLB manifests in a series of distinct yet related symptoms that comprise citrus responses to the bacterial infection. (A) Yellow shoot. Notice the HLB expression in sectors. Left lower section expresses no HLB symptoms. Right top section (arrow) expresses the typical yellow shoot symptom with thin canopy, branch dieback and reduced fruit load. (B) No symptom. Citrus leaves from HLB negative trees. (C) Blotchy mottle. Citrus leaves from HLB positive trees with yellow discolorations (yellow islands, arrows) appearing in non-symmetric patterns in relation to the leaf midvein. Starch accumulation and reduced number of chloroplasts has been reported in the mottled leaf areas. (D) No symptom. Citrus crop from HLB negative trees. (E) Citrus greening. Citrus crop from HLB positive trees with uneven fruit coloration and the reduced fruit size. (F) No symptom. Normally maturing citrus fruit with color break (orange) initiating at the stylar end progressing upwards to the green stem area of the fruit. (G) Color inversion. HLB affected fruit with color break disrupted (inverted) with stylar end (arrow) remaining green while the stem area of the fruit is already orange. (H) Lopsided fruit. Longitudinal section of deformed HLB affected citrus fruit. (I) Lopsided fruit and aborted seeds. Cross section of deformed HLB affected citrus fruit with aborted seeds (arrow). Photos by G. Vidalakis and S. Halbert.
HOST RESPONSES TO Ca. LIBERIBACTERS

The relationship among plant hosts and their associated microbes is essentially guided by the plant innate immunity system. This system can recognize the normally indispensable microbe-associated molecular patterns (MAMP; or PAMP in a pathogen circumstance), which is mediated by pattern recognition receptors (PRR) located on the surface of the host cell membrane. In the case of a pathogen, the recognition of PAMP activates PAMP-triggered immunity (PTI), manifested by cascades of mitogen-activated protein (MAP) kinase signaling, transcriptional induction of pathogen-responsive genes, production of reactive oxygen species, and deposition of callose reinforcing the cell wall at sites of infection, all of which contribute to prevention of further pathogen infection (Chisholm et al. 2006; Jones and Dangl 2006). In the course of evolution, successful pathogens have emerged by deploying virulence effectors that can contribute to pathogen virulence, most of which work by interfering with PTI. As a counteraction, plants have evolved cellular processes that can specifically recognize the effectors, either directly or indirectly, by producing disease resistance (R) proteins. The ability of recognizing effectors and deploying efficient counteractions is termed effector-triggered immunity (ETI), which is a reactivation of PTI that activates approximately the same set of defense responses but at an accelerated and potentiated manner. ETI results in disease resistance and, usually, a hypersensitive response (HR) with visible cell death at the infection site (Chisholm et al. 2006; Jones and Dangl 2006). By activating PTI or ETI, or a combination of both, plants can protect themselves from attack by many pathogens.

The exploration of the citrus defense responses to Ca. Liberibacter is relatively nascent to date. There might be several factors contributing to this situation. On one hand, genetic manipulation of the pathogen is very difficult: Ca. Liberibacters are notoriously well known for their uncultivable nature. On the other hand, characteristic host resistant responses such as a HR often are not apparent in the background of representative shoot or leaf yellowing. Historically this has been explained from an evolutionary perspective that HLB is a recent occurrence in evolution when compared with citrus planting (thousands of years) (Bové 2006; Gottwald 2010). It is conjectured that Ca. Liberibacters are of an animal or insect origin (possibly an insect endosymbiont), which first came in contact with citrus phloem via insect feeding activities about 100 years ago (Bové 2006; Gottwald 2010). Therefore, the host plants have not evolved sufficient immune responses to effectively ward off the infection (Gottwald 2010). This speculation is supported by the fact that so far no resistant citrus seedling
trees or scion-rootstock combinations have been identified. However, the long and variable incubation period of HLB symptoms development (Manjunath et al. 2008; Shen et al. 2013), and the fact that some plants are more successful than others, suggests that the plants are fighting the disease. In the past couple of years, progress on high-throughput sequencing and the recent availability of several Ca. Liberibacter genomes have advanced our ability to investigate host responses. To date, genomes from multiple Ca. Liberibacters species, including Las, Lam, Ca. L. solanacearum and Liberibacter crescens (the only Liberibacter that has been grown in axenic culture) have been sequenced (Duan et al. 2009; Lin et al. 2011; Leonard et al. 2012; Wulff et al. 2014). A lot of characteristic innate immunity elicitors and defense responsive components and signaling molecules have been identified in Ca. Liberibacters and citrus, respectively. Although the function and regulatory mechanism are not known yet, it is reasonable to compare these HLB-associated immune components with their homologs in other well-characterized immune systems (Durand et al. 2010) and assume their function in the citrus circumstance. From this aspect, the relationship between Ca. Liberibacters and citrus now can be interpreted from a plant innate immunity perspective, and recent progress on citrus responses to Ca. Liberibacters from PTI, a “potential ETI”, and metabolic aspects will be discussed in this session (Figure 3).

Citrus PTI responses

Genome sequence analysis indicates that Las can encode the bacterial flagellum component, flaglin (Fla), which contains a conserved 22 amino acid domain (flg22) at its N-termnus. Flg22 is a well-known PAMP that can activate plant defense mechanisms. When transiently expressed in tobacco, Fla was able to act as a PAMP and trigger host plant resistance, although to a lesser extent than other well-studied bacterial pathogens (Zou et al. 2012). The gene encoding Fla is absent in Lam; therefore, it is not clear at this time if there is a corresponding weaker, or even absence of PTI reaction in

Figure 3. A conceptual citrus innate immunity model

The up-to-date identified citrus immunity components (and their associated signaling, catalytic, and metabolic pathways) are projected to an overview of the current understanding of plant innate immunity, adapted from Panstruga et al. (2009) with modification. Reported citrus homologs of innate immunity components (and related pathways) are colored, while the yet unidentified components or unclear functions are depicted in grey. Solid lines indicate confirmed interactions, translocations or biosynthesis, whereas unconfirmed activities are indicated with dashed lines.
Ca. phytoplasma species that induces callose deposition in sieve plates and eventually destroys phloem function by causing necrosis and collapse of the sieve elements (Lee et al. 2000). The consistently observed callose deposition may suggest an important role it plays in citrus defense response.

When citrus is infected by Las, phloem protein 2 (PP2) is specifically induced (Albrecht and Bowman 2008; Kim et al. 2009; Mafra et al. 2013). PP2 is one of the proteins required for sieve pore plugging, and which has been suggested functioning as a defense response that may restrict further spread of Ca. Liberibacters within the sieve tubes (Musetti et al. 2010). However, the role induced PP2 plays in citrus defense is not conclusive, since PP2 is not induced at the early stage of Liberibacter infection. Instead, induction of PP2 might actually contribute to the clogged sieve tube, which further aggravates the HLB symptoms (Wang and Trivedi 2015).

In a citrus transcriptional analysis using microarray analyses, Aritua et al. (2013) identified some plant defense genes with homology to known receptor-like kinases (RLKs) in other plant species (Figure 3). Mafra et al. (2013) also observed induced expression of a LysM receptor like kinase (CERK1) and other RLKs. It’s intriguing that the expression of these RLK encoding genes are induced in Las-infected plants since RLKs are proteins localized to the surface of cells where these proteins should have no chance to get into contact with Liberibacters present within cells. The repeated observation of induction of RLKs suggest we should either reevaluate the assumption of the exclusive intracellular existence of Liberibacters, or there is a mechanism that host cells could relocate some PAMPs to the cell surface and present them to the RLKs, possibly for reinforcing the defense responses. It is also possible that there is an intermediate molecule between these intracellular localized PAMPs and the membrane anchored RLKs. This molecule could act as a decoy sensing the presence of PAMPs, while the molecule itself is monitored by RLKs (Figure 3). For example, decoy molecules

intermediating PAMPs and RLKs have been identified in tomato. Tomato RCR3 is the decoy in the AVR2/RCR3/Cf-2 signaling pathway where the membrane-localized receptor (Cf-2) guards the modification occurred to the decoy molecule (RCR3, a decoy of Pip1 that is a part of the tomato defense response), which is targeted by the avirulence factor (AVR2) from the fungal pathogen Cladosporium fulvum (Figure 3) (Shabab et al. 2008).

Citrus “potential ETI” responses

A gene-for-gene relationship is recognized as the central dogma to characterize a typical ETI. In this relationship, a secreted effector with contribution to pathogen virulence and the recognition of this effector by its cognate R protein leading to plant defense responses are the two key components. According to this criteria there is no clear ETI type resistance so far identified in the citrus-Liberibacters interaction system. This is in agreement with the current nonexistence hypothesis of resistant citrus seedling trees or scion-rootstock combinations.

However, varied tolerance to Liberibacter infection does exist in different genotypes. In a systematic survey conducted by Folimonova et al. (2009), the responses of 30 different genotypes of citrus grafted with Florida isolates of Las were examined. Based on the symptoms developed and the ability of plants to continue growth, the different genotypes were grouped into three categories: Sensitive, moderately tolerant and tolerant. When a HLB tolerant rough lemon is compared to a HLB sensitive sweet orange at the anatomical level within the leaf, stem and root tissues, fewer disruptive anatomical changes were observed in rough lemon than in sweet orange. However, this study supports the idea that there is no obvious anatomical change in HLB tolerant citrus that may contribute to their ability for sustaining plant growth after infection (Fan et al. 2013). There is no sign of any PTI responses being employed in these highly or moderately tolerant genotypes (Folimonova et al. 2009). Therefore, we could tentatively attribute the observed tolerance to a “potential ETI”, which have activated some responses resembling ETI: initiating large array of gene alterations, some of which are resistance-associated; inducing secretion of small anti-microbial molecules such as pathogen related proteins (PRs); activating/ suppressing plant hormones such as SA or JA that are critical in defense regulation (Figure 3) (Chisholm et al. 2006; Jones and Dangl 2006). To simplify the articulation of this concept, any citrus responses that meet these criteria will be referred to as a “potential ETI” reaction in this section.

Analysis showed that over 10% of the genes with significant altered expression after Las infection were related to plant defense and stress (Kim et al. 2009). These genes may participate in defense responses in a somehow direct manner, such as genes encoding nucleotide binding site-leucine-rich repeat (NBS-LRR) proteins, PR proteins, SA biogenesis or degradation modulators, and so on. There are also some factors involved in immune responses in a rather indirect manner, such as genes encoding member of the WRKY transcription factor and ethylene response factor subfamily members (Figure 3).

Many NBS-LRR family proteins participate in plant defense by recognizing pathogen-derived effectors directly, or indirectly by guarding a decoy molecule (Chisholm et al. 2006;
Among these affected genes, the expression of a gene encoding a TMV N-like disease resistance protein, and an NBS-LRR-like protein Cdc7, were down-regulated. Other resistance-related genes showing similar patterns include a putative TIR-NBS-LRR-class protein, and an inter-alpha-trypsin inhibitor heavy chain-related protein (Aritua et al. 2013). The same study also identified NBS-LRR genes showing an opposite expression pattern, including the gene encoding a CC-NB-LRR domain containing protein, a resistance protein candidate 2 (RGC2), and a disease resistance family protein SCOA belonging to the Cladosporium fulvum resistance protein Cf-9 (Hcr9) family (Aritua et al. 2013). It’s intriguing that in the same plant infected with Liberibacters, some disease resistance genes showed enhanced expression while others showed reduced expression. It is possible that Liberibacters are capable of secreting some effectors into the host cells and subverting the host defense responses by manipulating the expressions of some critical defense-related genes.

In accordance with the above speculation, salicylic acid (SA) signaling might be influenced by Ca. Liberibacter infection. In Las-infected Valencia plants, SA accumulated to about two-fold when compared to healthy plants (Lu et al. 2013). SA is a key plant hormone regulating plant immune responses, and a candidate for long-distance signal transmission in systemic acquired resistance (SAR). Induction of SA in citrus leaves indicates an elevated host response has been induced by the Las infection, and a SAR may already have been primed to the unaffected tissue. On the other hand, Las can encode a salicylate hydroxylase that converts SA into catechol, a product that does not induce resistance (Aritua et al. 2013). Therefore, it appears that Las may use salicylate hydroxylase as a mechanism to evade plant defense. Besides the yellowing of shoots and leaves that may mask the visibility of a HR associated with ETI, the counteraction from Las by manipulating host SA accumulation might be another reason for the inconspicuousness of HR.

Liberibacter infection affects expression of many genes the encoding proteins of which contribute to plant innate immunity by different mechanisms. For instance, synthesis and secretion of PRs are markers for an activated plant ETI response. PR3 and PR4 are induced in sweet orange after Lam infection (Mafra et al. 2013). The transcription of PR-10 is elevated in Las-infected stems but not in roots (Aritua et al. 2013). These corresponding regulation patterns of expressed PR genes indicates that citrus hosts can recognize and react to the invasion and infection of pathogens in a similar pattern employed by other plants (Kim et al. 2009). Expressed miraculin-like proteins have also been identified in several independent studies. The up-regulation of miraculin-like proteins was observed either at the transcriptional (Aritua et al. 2013) or translational levels (Nwugo et al. 2013a, 2013b). The miraculin-like proteins possess an endopeptidase inhibitor activity hydrolyzing nonterminal peptide bonds in polypeptides that stops, prevents or reduces the activity of an endopeptidase. Homologs in soybean can specifically inhibit the activities of membrane-bound serine proteases, which are multifunctional enzymes manipulating functions of protein precursors and biologically active factors (Zamolodchikova 2013). Functionally, miraculin-like protein can contribute to innate immunity by modulating serine protease activities.

Las infection also affects the expression of genes encoding proteins containing the WRKY domain, such as WRKY4, WRKY23 and WRKY30. Many WRKY domain-containing proteins are involved in plant defense responses. Overexpression of WRKY4 enhanced susceptibility of Arabidopsis plants to P. syringae and suppressed PR1 gene expression, but the effect of up-regulation of WRKY4 in Las-infected citrus remains to be addressed (Aritua et al. 2013). Citrus homologs of WRKY6 and WRKY40 were also up-regulated at the transcriptional level in sweet oranges infected with Lam (Mafra et al. 2013).

These observed “potential ETI” responses summarized above clearly demonstrate that there must be a functioning “effector-R protein” interaction occurring in citrus cells upon Ca. Liberibacter infection. However, no effectors with sequence or domain similarity to currently identified effectors in other plant pathogens have been identified in Las (Wulff et al. 2014). So far most of the well-studied effectors are type III secretion system (T3SS)-secreted effectors. Analysis of Liberibacter genomes indicates that neither Las nor Lam encode any T3SS components. Instead, genes encoding a type I secretion system (T1SS) are present in Ca. Liberibacters. Similar to T3SS, T1SS also employs an one-step transport mechanism that can secret proteins varying greatly in size and function. In animal pathogens, many proteins secreted via the T1SS are of great importance for the pathogenesis in the host organism, or for antibacterial activity (Kanonenberg et al. 2013). It’s reasonable to propose that although a classic effector delivery system (such as T3SS) has not been identified, a similar mechanism (such as T1SS) may be operating and delivering pathogenesis-related effectors to the cytoplasm of the host cells, considering the intracellular nature of Liberibacters (Duan et al. 2009; Wulff et al. 2014). A recent study identified 14 adenosine triphosphate-binding cassette (ABC) transporter systems encoded by the Las genome, some of which might be involved in secreting virulence factors (Li et al. 2012). One of the ABC transporter systems identified is predicted as a classic T1SS, and the possible substrate of this T1SS is a RTX (repeats in toxin) protease serralin. Serralin metalloprotease secreted by Serratia marcescens can suppress cellular immunity of silk-worms by decreasing the adhesive properties of immune surveillance cells, thereby contributing to bacterial pathogenesis (Ishii et al. 2014). Therefore, it is very likely that in an infected citrus plant, there are interplays between the citrus innate immunity and the Liberibacter T1SS-secreted effectors, which shapes the disease development in plant tissues (Figure 3). With the advance in the functional study of Ca. Liberibacter genomes, we speculate more “non-canonical” effectors like serralin will be identified.

**Diverse metabolic responses**

Liberibacter infection causes accumulated starch in the sieve elements, ultrastructural changes of phloem tissue, plugged sieve pores, and eventually disrupted phloem. In citrus cells, many metabolic activities can affect HLB symptoms. For example, starch accumulation is related to metabolic processes such as photosynthesis, respiration and energy availability. Phloem aberrance is related with callose

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*Jones and Dangl 2006*. In multiple studies comparing healthy and Liberibacter-infected citrus, the expression of several NBS-LRR genes were predominately affected (Kim et al. 2009; Aritua et al. 2013; Mafra et al. 2013; Nwugo et al. 2013a, 2013b).
deposition, PP2 accumulation, and cell wall synthesis, assembly and modification. Therefore, close monitoring on certain metabolic processes may shed light on alternative citrus responses that can efficiently defend a Liberibacter infection.

Study in Populus indicates that respiration and glucose catabolism-related proteins are up-regulated, while proteins involved in photosynthesis are down-regulated under stress conditions (Durand et al. 2010). This is in agreement with observations in Liberibacter-infected citrus plants, where up-regulation of carbohydrate synthesis elements and down-regulation of photosynthesis components are frequently observed (Albrecht and Bowman 2008; Kim et al. 2009; Aritua et al. 2013; Nwugo et al. 2013a, 2013b). Multiple reports have shown that expressions of many genes in which protein products are involved in carbohydrate metabolism are affected by Liberibacter infection. Proteins that participate in starch synthesis (adenosine diphosphate-glucose pyrophosphorylase large subunit 3) and starch granules synthesis (GBSS) were up regulated by infection (Kim et al. 2009; Aritua et al. 2013; Nwugo et al. 2013a, 2013b). Also observed were the down-regulation of genes encoding beta-amylase 1 (BMY1) and a neutral invertase involved in the degradation process. The synergistic gene expression alteration leads to accumulation of sugar and starch in the infected tissue. Whether the elevated starch level is an active response to infection is not yet known.

As observed in other plant species, photosynthesis generally is suppressed during stress conditions when resources are channeled to defense-related responses. This also is true in citrus. Photosynthesis pathway components from either photosystems I or II are down regulated in Liberibacter-infected citrus, which has been reported in different citrus species (sweet orange, grapefruit and lemon) at both transcription and protein levels (Albrecht and Bowman 2008; Nwugo et al. 2013a, 2013b). It should be noted that the accumulation of carbohydrate and starch could also result in an inhibition of photosynthesis via a negative feedback mechanism. Therefore, whether the inhibited photosynthesis is an active response against Liberibacter infection, or a passive result due to the negative feedback, or a combination of both, needs further investigation.

Phloem cell deformation at the tissue level and reduced shoot and root growth at the tree level are associated with HLB. In diseased plants, cell wall integrity is an important battlefield in the host and pathogen combat. Upon Liberibacter infection, expression of some genes encoding proteins involved in cell wall synthesis, assembly and modification are altered. For example, genes involved in cell wall assembly (such as a proline-rich protein) and cell wall extension (such as expansin-related protein 1) are specifically up-regulated at the transcriptional level in Las-infected tissue (Aritua et al. 2013). In the same tissue, genes encoding enzymes involved in the hemicellulose backbones and cellulose synthesis are repres sively expressed. Again, whether the alteration over cell wall integrity components is an active action or a passive consequence needs more detailed examination.

Citrus immune reaction is a highly integrative response
As summarized above, upon Liberibacter infection, citrus plants react in a potent way that can both counteract the deleterious effect imposed by the pathogen infection, and prevent disease from further development. Resistant responses employed include both typical PTI reactions such as induction of RLKs and callose deposition in affected tissues, and classic ETI responses such as induction of NBS-LRR and PR proteins, and SA elevation (Figure 3). It’s reasonable to speculate that there may be master machinery that can smoothly maneuver this sophisticated defense tool kit. Studies in Arabidopsis showed that plant innate immunity could be cognately regulated by a pair of microRNAs (miRNA), miR393 and miR393b* that modulate PTI and ETI, respectively (Zhang et al. 2011; Niu et al. 2015). Since miR393 and miR393b* are generated from the same precursor that undergoes an identical expression regulatory mechanism, the two branches of the innate immunity are now under adjustment to a single stress cue. Similar regulatory mechanisms have not yet been identified in citrus. However, small RNAs (smRNAs) playing a regulatory role in citrus innate immunity have been identified. When smRNA expression profiles from healthy and Las-infected citrus sinensis were compared, panels of smRNAs that are differentially expressed were identified. One of the specifically induced miRNAs, miR399, can regulate phosphorus homeostasis by targeting a key phosphorus transport modulator, PHO2 (Zhao et al. 2015). This study further suggested a relationship between phosphorus deficiency and HLB disease.

With the set of integrative immune responses implemented, citrus plants should be able to ward off threats imposed by Liberibacter infection. The effectiveness of citrus defense responses is sometimes obscured by the delusion that HLB is so destructive that there is no room for innate immunity to play. Etiologically, most of the deleterious symptoms associated with HLB can be attributed to the disrupted phloem (Bové 2006; Kim et al. 2009; Gottwald 2010; Aritua et al. 2013; Nwugo et al. 2013b). Microscopy imaging showed that Las does not form aggregates in citrus phloem; rather, plugged sieve pores are caused by callose deposition, induction of PP proteins, accumulation of sugar and formation of starch granules (Kim et al. 2009; Folimonova and Achor 2010). Further pathogenesis studies will not only illustrate the mechanism(s) of disease formation and development, but also demonstrate the potential effectiveness of innate immune against the HLB disease.

POPULATION BIOLOGY OF ‘CA. LIBERIBACTER’ SPECIES
Molecular markers are important to study the epidemiology of economically important species to track changes in species or genotypes over time which can lead to better disease management strategies (Milgroom and Peever 2003). For Ca. Liberibacter species, most published work in this area has focused on Las since it is the most important species associated with the destructive HLB disease of citrus and has widespread occurrence in the Western Hemisphere. While L. africana has been associated with the citrus greening disease in Africa due to the environmental requirements of its psyllid vector T. erytreae, that is, high elevation and low temperatures, and L. africana’s sensitivity to heat and low humidity, the Laf citrus greening has proven less destructive and relatively
easier to manage in comparison to the Las-associated HLB (Aubert 1987; Bové 2006). In addition, since the discovery of Lam in Brazil in 2005, its occurrence decreased from 98 to 20% in a period of less than 4 years. Lam also has a competitive disadvantage over Las due to lower graft-transmission efficiency and in the concentration of bacterial cells in citrus plants. Therefore, Lam most likely plays a less significant role in the HLB epidemic in Brazil in comparison to Las (Teixeira et al. 2005a, 2005b; Lopes et al. 2009).

The first laboratory-based studies to investigate Ca. Liberibacter species variability were based upon monoclonal antibodies. The main emphasis for these early studies was to try to find a technique to identify these bacteria since they could not be cultured. For example, Garnier et al. (1987) developed monoclonal antibodies that could detect both Las and Laf infected citrus material in hopes of developing a diagnostic procedure for the greening disease. Latter studies also used monoclonal antibodies and were able to detect isolates from different geographic areas but none of the antibodies could detect all Ca. Liberibacter isolates, providing some early insight that genetic variability existed within these species (Garnier et al. 1991; Gao et al. 1993). These early studies also determined that at least seven distinct “serotypes” could be detected using serological methods, providing further evidence of genetic variability within these bacterial species (Gao et al. 1993).

DNA-based methods were the obvious next step toward understanding HLB isolate variability, but the early studies, as emphasized above, were primarily aimed at developing detection methods as well as methods to identify specific bacterial species associated with HLB. Ribosomal regions, both partial 16s rDNA and 16s/23s rDNA spacer regions, were the first regions to be investigated since these regions could be polymerase chain reaction (PCR) amplified, and have been routinely used in species and strain identification of bacteria (Villechanoux et al. 1993; Jagoueix et al. 1997; Kolbert and Persing 1999). In these studies, both regions were found to be too conserved for isolate identification and were, therefore, only useful in determining species (Bastianel et al. 2005). For example, many Japanese isolates and several Southeast Asian isolates from Taiwan, Indonesia, the Philippines, Vietnam, and Thailand were shown to have identical 16s rDNA sequences (Subandiyah et al. 2000; Tomimura et al. 2009). However, Adkar-Purushothama et al. (2009) found much more sequence variation within 16s rDNA from primarily Chinese isolates of Las and suggested this locus could be used for population genetics studies. In this study, 14 single nucleotide polymorphism (SNP) 16s lineages were identified, but there was little bootstrap support among clades and each “lineage” differed by, at most, two SNP markers.

A major hurdle for studying the population biology of the HLB pathogens, besides not being able to work with bacterial cultures, was that none of the molecular markers developed were very useful to characterize populations of HLB species. In an attempt to develop a better marker system, Hocquillet et al. (1999) used variation in random amplified polymorphic DNA (RAPD) profiles from HLB infected and non-infected plant material. They isolated, cloned and sequenced DNA fragments that were present only in the HLB infected profiles and identified four genes; nusG, pgm, omp and an unidentifiable hypothetical protein gene with the hope that they may be potentially useful molecular markers. Subsequently, Bastianel et al. (2005) used a PCR-restriction fragment length polymorphism approach to study Las isolates based on the omp gene and found that they clustered into two groups: isolates from India and Nepal and isolates from Thailand, the Philippines, and China. Hu et al. (2011) also used the omp gene to study the diversity of Las isolates from China and found that the isolates clustered into three subgroups. Both studies found evidence of potential clustering based on geography but both studies suffered from very low bootstrap support for the clades found and small sample size: nine and 23 isolates, respectively. In contrast, Deng et al. (2008) found little variability within the omp gene from Las isolates from China collected only from pomelo and suggested it was likely due to a recent introduction into the production area where the isolates were sampled.

Villechanoux et al. (1992) used differential hybridization techniques to develop DNA-based probes that were able to differentiate between Las and Laf isolates as well as detecting polymorphisms within one probe for various Las isolates. Some of these probes were later characterized and determined to be partial genes of rpo/KAJL-rpoBC operon (ß operon), nusG and bacteriophage-type DNA polymerase genes (Villechanoux et al. 1993; Planet et al. 1995). Tomimura et al. (2009) sequenced the bacteriophage-type DNA polymerase locus from Southeast Asian isolates of Las and found three well-supported clades; one clade contained only Indonesian isolates but the isolates from the two other clades did not correlate with geographic origin. The rpo/KAJL-rpoBC gene cluster was further characterized in additional studies and extended to include the VserA-trmU-tufB-secE-nusG-ß/KAJL-rpoBC gene cluster plus flanking sequences (Okuda et al. 2005; Lin et al. 2008; Furuya et al. 2010). This 11,168 bp region was later used to study the diversity of Las isolates primarily from Japan. Despite sequencing a relatively large amount of nucleotides, only 11 SNPs were found from 62 isolates from Japan, Taiwan, Indonesia and Vietnam. Twelve genotypes were identified but 42 out of 62 were identical; only two SNPs were reciprocally fixed between the isolates with no obvious geographic pattern, and the remaining genotypes only differed from one another by one to two SNPs (Furuya et al. 2010).

Research on Las genomics has provided researchers with complete sequence data to develop more sensitive molecular markers to study the population biology of the HLB pathogens (Duan et al. 2009; Tyler et al. 2009). Chen et al. (2010) were the first to take advantage of this resource and identified a locus containing a single variable tandem repeat; that is, a microsatellite locus. They investigated the diversity of isolates from Guangdong, China and Florida, USA where the disease was first observed approximately 100 and 15 years ago, respectively. Based on this single locus, nine genotypes could be detected based on repeat number, and most isolates had different length differences between the two countries. However, some genotypes were shared between countries and more diversity was found within China. Zhou et al. (2011) used two variable tandem repeats (hyv and hyvα) within the prophage regions of Las. Isolates from Florida contained both hyv and hyvα, while all other global Las isolates contained either one or the other, and they hypothesized that there was a ‘multisource’ introduction into Florida. Hyv and hyvα were
Las has been found in all stages of *TRANSMISSION OF LIBERIBACTERs* and were, therefore, renamed as *las*A1 and *las*A2 (Hao et al. 2013). Puttamuk et al. (2014) also used these markers to study a large collection of infected citrus and ACP (almost 300 samples), primarily from Thailand, and found significant diversity. They hypothesized that the pathogen was likely introduced to Thailand from China and/or the Philippines based on their phylogenetic analyses.

Matos et al. (2013) used four microsatellite markers to investigate diversity from HLB isolates from Florida, USA, Mexico and various parts of the Caribbean. Similarly to Chen et al. (2010), lower genotypic diversity was found in Florida compared to the other regions sampled where the disease has been prevalent for a longer period of time. Katoh et al. (2011) also used four microsatellite markers to analyze isolates primarily from Japan. Eighty-four isolates could be differentiated using these markers, and cluster analysis divided the 104 isolates studied into 10 major clusters that were primarily correlated with the geographic origin of the isolates. However, no bootstrap support values were presented for these ‘major clusters’. Katoh et al. (2012) used 13 polymorphic microsatellite markers as well as 39 SNPs located ≈200 bp away from microsatellite locus 091 to study the diversity of isolates from India, East Timor, Papua New Guinea and Florida (n = 24). Cluster analyses of both sets of markers were similar, and isolates grouped primarily by geographic origin as was found in earlier studies.

To the best of our knowledge, the most comprehensive study to date based on a combination of sample size, number of loci and analytical methods used was conducted by Islam et al. (2012). In this study, seven microsatellite markers were developed and used to characterize 287 isolates of Las collected from Florida, Brazil, China, Cambodia, Vietnam, Thailand, Taiwan, Japan and India. UPGMA (unweighted pair group method with arithmetic mean) analysis revealed three main clades of Las, but as with previous studies mentioned above, the dendrogram had low to no bootstrap support for the identified clades. However, one clade contained primarily Brazilian and East-Southeast Asian isolates, another clade contained primarily Florida isolates, and the third clade contained only isolates from India. The Bayesian modeling approach used in the program STRUCTURE to assign individuals to populations with no prior knowledge of where they were sampled, also supported the findings of the UPGMA analysis. Islam et al. (2012) hypothesized that at least two introductions into Florida have occurred since some isolates from the East-Southeast Asian clade and Brazilian clades were found within the primarily Florida group and that the three globally identified groups identified were likely the founding populations of Las globally. This also was supported by an additional networking-based analysis conducted by Islam et al. (2012).

### TRANSMISSION OF LIBERIBACTERs

Las has been found in all stages of *D. citri*, its most common vector (Pelz-Stelinski et al. 2010). Xu et al. (1988) were able to find the bacteria in both salivary glands and the mid-gut of the psyllid. The details about transmission parameters are variable and complex, probably depending on whether acquisition and transmission take place via single individual adults or within a colony. Early experiments were conducted by collecting adult psyllids from infected field sites, or caging them on infected plants for a period of time, and subsequently transferring them to healthy test plants. Before the days of molecular diagnostics, the only means of assessing transmission was to observe plants for symptom development. A good review of the early information can be found in Pelz-Stelinski et al. (2010). Researchers rarely controlled for or made notes on whether the insects were allowed to colonize the infected plants or the test plants.

Xu et al. (1988) did extensive studies on transmission parameters. The impetus for the experiments came from their field observations. Up to 70% of citrus trees became infected prior to bearing age in plantations in Guangdong and Fujian provinces, even though transmission efficiency was reported to be low. Xu et al. (1988) reported transmission by fourth and fifth instar nymphs, but not by first, second or third instars. They found a minimum positive inoculation access period of 5 h by adults that had been raised on infected plants. In serial transfers of single adults, they found transmission to healthy plants after up to 13 transfers. Transmission appears random (see their table 1), with some insects transmitting to more plants early in the process, and others transmitting only to the latter plants. Every insect infected at least one plant, even the 10 psyllids that were allowed access to an infected plant only for 2 d, and only as adults. No notes were made about whether colonies were allowed to form on the test plants. (Presumably, half of the test psyllids were male, and so in those cases, no colonies could form).

In contrast to Xu et al. (1988), Inoue et al. (2009) obtained no transmission at all by psyllids given access to infected plants only as adults. Furthermore, the percentage of psyllids testing positive declined over time until it reached 50% after 20 d. There was no increase in the concentration of the pathogen in the positive psyllids. Pelz-Stelinski et al. (2010) also found a decline in percent *D. citri* testing positive over time on healthy plants. If Las was acquired by nymphs, the percentage of positive psyllids was maintained over time, and the concentration of the pathogens increased over time, suggesting multiplication in the nymphs. Psyllids that were given access to Las as nymphs infected 65% of the plants in transmission experiments.

Pelz-Stelinski et al. (2010) confined adults on field trees and on laboratory plants. Since the access periods exceeded 35 d, presumably the psyllids were allowed to colonize the plants. In both cases, increases in numbers of positive psyllids occurred at each weekly sampling period. Only 5% of single adults successfully inoculated citrus plants. There was no difference in efficiency as the inoculation access period was increased. Presumably the females given longer inoculation access periods were allowed to colonize the plants.

The status of transovarial transmission of Liberibacters in psyllids has been a subject of debate for a long time (Halbert and Manjunath 2004). Pelz-Stelinski et al. (2010) found a small amount of transovarial transmission. Mann et al. (2011) found a small amount of sexual transmission, but only from infected males to healthy females. This is not too surprising, in that the best explanation of the results obtained by van den Berg et al. (1992) (see discussion in Halbert and Manjunath (2004)) is transovarial transmission of LaF in *T. erytreae*. Moreover, Hansen et al. (2008) showed transovarial transmission of *Ca. L.* psyllaurus.
A novel transmission mechanism was discovered by Lee et al. (2015). They placed 3-10 small healthy citrus plants in a cage, and added 50 D. citri that were 20%–70% positive for Las. After 15 d all the original adults were removed but their nymphs were left on the plants. At 30 d, the adult progeny of the original psyllids were harvested. The results were variable, but these insects were 5%–83% positive for Las. Not all the plants were colonized by the psyllids, but those that were colonized usually developed symptoms months after the psyllid experiments were completed. The shoots where the psyllids had colonized were tested, and these were also positive. Thus, it appears that an adult psyllid, probably infected as a nymph, infects the new citrus growth (flush) where the female lays her eggs. As the nymphs develop, they acquire Las, which multiplies in their bodies. As soon as adults emerge, they are able to repeat this process. Since a female psyllid lays up to an average of 748 eggs under optimum conditions, the potential for increase in numbers of positive psyllids is enormous (Tsai and Liu 2000).

McClean (1974) found similar results in a series of experiments where he tested adult T. erytreae that emerged from nymphs in a prior series of transmission experiments (see his table 3). The second-generation adults transmitted greening to multiple plants in three cases where there was no detectable disease in the first cycle of experiments.

Chiyaka et al. (2012) defined the latent period for a pathogen as the time between infection and the time that the pathogen is accessible to another vector. The incubation period is the time between infection and the development of disease symptoms. For most pathogen systems, these terms have been used interchangeably, because the pathogens become accessible to vectors at about the same time that symptoms develop. Given this new transmission mechanism, the latent period and incubation period for HLB can be vastly different. The latent period is as short as a single generation of psyllids (15 d), whereas the incubation period can stretch for at least 6 years (Shen et al. 2013). This mechanism may explain the early observations of Xu et al. (1988), and also the rapid incursion of HLB in localities such as Florida, where it has been introduced.

Psyllids have been tested for Liberibacter species for a long time (Bové et al. 1993), but psyllid testing was done only to confirm presence of the bacteria in the vectors. Manjunath et al. (2008) were the first to use psyllid testing to look at large-scale epidemiological questions and as a result, a lot has been learned about the distribution and movement of HLB in Florida (Halbert et al. 2010; Halbert et al. 2012). Las could be found in psyllids months before symptoms appeared on infected plants. Records for positive psyllids in a given locality sometimes preceded discovery of Las in local plants by several years (Manjunath et al. 2008). Positive psyllids were found traveling on oranges in fruit trailers. The insects were distributed throughout the loads, and they were on the fruit itself and not on accompanying vegetative debris (Halbert et al. 2010).

Finally, testing psyllids has proven valuable in assessing the status of plants for sale (Halbert et al. 2012). Psyllids testing positive for HLB were found an average of 9 months prior to the discovery of positive-testing symptomatic plants in retail venues. Halbert et al. (2012) tested psyllids sent in for regulatory confirmation to the diagnostics bureau at the Florida Department of Agriculture and Consumer Services, Division of Plant Industry (DPI). Testing was carried out over a period of 4 years, and nearly 1,200 regulatory samples were analyzed. Overall, approximately 10% of these samples were positive for Las. In 2008, about halfway through the sampling period, new regulations were enacted by DPI that required regular monthly inspections and enclosed structures for all phases of citrus propagation (but not retail sales). As a result, the percentage of psyllid samples from citrus propagation facilities dropped dramatically in 2008 and remained low in the final year of the project (Halbert et al. 2012). The new transmission mechanism described in Lee et al. (2015) explains the high numbers of positive psyllids in retail environments. In order to multiply unimpeded, the pathogens need an unlimited supply of healthy new sprouting plants and a resident positive psyllid population. In this retail environment, there is ample opportunity that each newly arriving healthy plant could be colonized by resident psyllids, and by Las, which multiplies in the flush and infects the next generation of psyllids. Testing psyllids is an excellent way to determine if plants for sale are compromised by Las. Based on experiments reported by Lee et al. (2015), it might also be possible to test colonized new growth to obtain plant positives for regulatory purposes. Alabi et al. (2014) also used positive psyllids, with conformational plant testing, to assess an unprotected nursery in Texas.

Much remains to be learned about when and how psyllids become positive, and similarly when and how they become competent vectors. Recent surveys of populations in Florida indicate great variability in the infection rate of field populations of D. citri (Coy and Stelinski 2015).

**ECOSYSTEM JUMPING**

It is not clear how Liberibacter species move from one psyllid/plant ecosystem to another. It is unlikely that associations among citrus and the three Liberibacter species that are associated with HLB disease are original associations (Beattie et al. 2008). In solanaceous crops, psyllid yellows of tomato have been known since 1928, while zebra chip of potato, associated with Ca. L. solanacearum, is a recent pest problem (Richards 1928; Hansen et al. 2008; Liefting et al. 2009; Fathi 2011). The association between Ca. L. solanacearum and Umbelliferae also is new (Alfaro-Fernandez et al. 2012). In the case of Ca. L. europaeus and pear, the association of the bacteria with the Cacopsylla pyri (L) vector is most likely original, because the bacteria behave as endophytes rather than pathogens (Raddadi et al. 2011). On the other hand, the association between Ca. L. europaeus, scotch broom (Cytisus scoparius), an invasive leguminous exotic shrub, and the psyllid (Arytainilla spartiophila (Forster)) imported into New Zealand to control scotch broom, clearly is not an original one (Thompson et al. 2013). The association between L. crescens and mountain papaya is unlikely to be an original association, because Babaco (mountain papaya) has no known psyllids.

Thus, most of the known associations between Liberibacter species and crop plants (or weeds in the case of scotch broom) are acquired associations. There are several possible mechanisms for transfer. Dodder infection cannot be discounted, because the parasitic weed is common in agricultural landscapes, especially in potatoes, but it also can be found in other...
The pathogens do not have complete specificity with respect to psyllid vectors, in that both *D. citri* and *T. erytreae* can transmit *L. asiaticus* and *L. solanacearum* (Massonie et al. 1976; Lallemant et al. 1986). Thus, chance feeding could transfer the bacteria to a crop plant with its own psyllid pest, which then would perpetuate the infection in the crop. The chance discovery of L. crescens in Babaco (mountain papaya) supports this hypothesis, because it has no associated psyllid. On the other hand, psyllids associated with a crop plant could acquire the pathogens by feeding on wild plants with associated species of Liberibacters, carrying them back to the crop plants. Associations of *Ca. L. solanacearum* in various Umbelliferae in Europe are worrisome in this regard, because many vegetable crops there have associated psyllids in the *Bactericera nigricornis* (Förster) complex. One of these species, *Bactericera trigonica* Hodkinson is a proven vector of *Ca. L. solanacearum* in carrots (Alfaro-Fernandez et al. 2012). These three species are widely polyphagous with overlapping host ranges that include a number of crops (Hodkinson 1981).

**SPREAD OF HLB**

Spread of HLB occurs by insect vectors, by propagation (van Vuuren 1993) and experimentally by dodder (Ghosh et al. 1977; Tirtawidjaja 1981). Tirtawidjaja (1981) found results suggestive of seed transmission, based on symptoms in seedlings, but Hartung et al. (2010) and Hilf (2011) found no evidence of seed transmission in hundreds of tested seedlings from seed collected from symptomatic fruit. Spread of HLB must be examined on several scales. Spread within a tree has been modeled by Chiyaka et al. (2012). Spread within a tree is not uniform, indicated by relative number of infected grafts from symptomatic and asymptomatic portions of the tree (Lin and Lin 1990). van Vuuren (1993), working on Laf, made careful observations about the consequences of vector infection. He observed several outcomes: Shoots grew normally, the growth point where the insects fed died and subsequent growth was normal, terminal growth with severe symptoms developed, the terminal growth died, and lateral growth either developed severe symptoms or developed normally, symptomatic feeding area died back and subsequent growth was asymptomatic. However, sometimes, even after the initial symptomatic portion of the tree was removed, symptoms returned in 12 months. From these observations, it can be surmised that plant defenses occasionally can overcome initial infections through hypersensitivity and dieback. These studies led van Vuuren (1993) to conclude that citrus actually is a poor host of Laf. He also suggests that re-infection is important for maintaining disease, a conclusion also supported by Stansly et al. (2014).

Spread within a grove has been studied extensively using models that analyze spatial and temporal distributions of symptomatic trees (Gottwald 2010). All these models make the assumption that a tree that develops symptoms first also got infected first. This assumption holds if the incubation period is constant within a planting. However, if there is a dosage effect (numbers of infective psyllids?) or a micro-environment effect that also would cause one tree to develop disease before another one, these assumptions must be understood with caution.

Long-range spread of HLB is even more complex and involves human-assisted as well as natural spread. Lewis-Rosenblum et al. (2015) and Tiwari et al (2010) used marker proteins to mark large numbers of *D. citri* adults and assessed where they moved. Tiwari et al. (2010) found that psyllids tended to migrate from abandoned plantings into managed groves. Lewis-Rosenblum et al. (2015) elaborated on this work to show that movement was greatest during the spring and summer and that the psyllids were able to disperse at least 2 km within 12 d, unhindered by barriers such as fallow fields and highways. Human-assisted movement occurs via plants for sale (Halbert et al. 2012) and transportation, especially movement of unprocessed fruit (Halbert et al. 2010). The rapid spread in Florida probably cannot be accounted for by natural spread of infected psyllids alone.

**CONCLUSION AND FUTURE PERSPECTIVES**

Management of HLB to enable the continued economic production of citrus is the largest challenge ever faced by the citrus industry, worldwide. In areas such as China, Brazil, Florida where HLB is widespread, the challenge is to maintain what production is possible from the established, HLB infected trees and how to devise approaches that enable new plantings of citrus to come into production. In areas such as Texas, where HLB currently is spreading, and in Arizona and California where the ACP vector is present but the disease apparently has not been established, the emphasis is more on early detection, eradication and limiting the spread of the disease. With the emerging evidence supporting the idea that host responses may play a role in shaping HLB development, management employing host defense mechanisms should no longer be ignored.

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**AUTHOR CONTRIBUTIONS**

All authors contributed equally to this review. Mention of a trademark, warranty, proprietary product, or vendor does not imply an approval to the exclusion of other products or vendors that also may be suitable.

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