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MOLECULAR INTERACTION BETWEEN *CANDIDATUS LIBERIBACTER ASIATICUS* AND CITRUS: A REVIEW

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ABSTRACT
Huanglongbing (HLB), the most devastating disease of citrus, is associated with infection by *Candidatus Liberibacter asiaticus*, a phloem-limited, fastidious α-proteobacteria in the family *Rhizobiacea* and is vectored by the Asian citrus psyllid *Diaphorina citri Kuwayama*. In this study, the molecular basis of citrus-HLB interactions has been studied using transcriptome analyses, and these analyses have identified many pathways modulated by *C.* las infection among different citrus cultivars. Pathogen-associated molecular patterns (PAMP) activity of flg22 in Las is weaker than those in other well studied plant pathogenic bacteria. Predecessors thought that host citrus plants have not evolved sufficient immune responses to effectively prevent infection. The accessibility of high throughput sequencing, transcriptomes and proteomes have functional to Las-infected plants and treated plants; as a result, numerous characteristic innate immunity elicitors, transcription factors, defense responsive components, and signaling molecules have revealed. This aspect, the relationship between *Ca. Liberibacters* and citrus now can be interpreted from a plant innate immunity perspective. As a counteraction, plants have evolved cellular processes that can specifically recognize the effectors, either directly or indirectly, by producing disease resistance (R) proteins. HLB strongly affected pathways and processes, such as sugar and starch metabolism, cell wall metabolism, stress response, hormone signaling and phloem genes were significantly altered in citrus. This study helps to understanding some aspects related interaction between *Candidatus Liberibacter asiaticus* and citrus and helps to developing defense against Huanglongbing in future.

KEYWORDS: Huanglongbing (HLB)**,** *Candidatus Liberibacter asiaticus (*CLas), Transcriptome analyses, Pathogen associated molecular patterns (PAMP) and Effectors.

INTRODUCTION

Huanglongbing (HLB), also known as "citrus greening" is a devastating disease of citrus, which is distributed in citrus-growing areas worldwide, ranging from Asia to neighboring islands to Saudi Arabia, Africa, and America (Bové, 2006). Although first reported in China in 1919, HLB was likely established in India much earlier (Gottwald, 2010). Huanglongbing was found in Florida in 2004 (Halbert, Manjunath), after one year its first discovery in Western Hemisphere, Brazil (Teixeira *et al.*, 2008). Typical symptoms of disease are an asymmetric blotchy mottling of leaves (McClean and Schwarz, 1970), often resembling zinc-deficiency symptoms, which leads to the typical appearance of yellow shoots in the tree canopy. The causal agent of HLB is a fastidious, phloem-limited, Gram-negative bacterium (Mubeen *et al.*, 2015a, b; Garnier *et al.*, 1983), which has not been obtained in pure culture until recently (Damsteegt *et al.*, 2009). Three *Candidatus* spp. of the pathogen are currently known. The most widespread Asian species, *Ca. Liberibacter asiaticus*, is found in all HLB-affected countries except Africa. The African species, *Ca. L. africanus*, and the American species, *Ca. L. americanus*, are so far restricted to Africa and Brazil, respectively (Garnier *et al.*, 2000). Transmission of the bacteria occurs through insect vector, but while *Ca. L. asiaticus* and *Ca. L. americanus* are transmitted through *Diaphorina citri Kuwayama*, the Asian citrus psyllid, *Ca. L. africanus*, is transmitted through the African citrus psyllid, *Trioza erytrea*. In addition, transmission can occur by dodder (*Cuscuta sp*.) and through grafting with diseased budwood (Halbert and Manjunath, 2004). Methods such as electron microscopy, serology, DNA probes, enzymatic assay, enzyme linked immunosorbent assays (ELISA), conventional polymerase chain reaction (PCR) and quantitative PCR (qPCR) are used for the diagnosis and confirmation of HLB (Kogenaru *et al.*, 2014). X-ray fluorescence (XRF) and laser-induced breakdown spectroscopy (LIBS) combined with chemometric strategies are used to successfully predict the condition of orchard plants infected with *Candidatus liberibacter* spp. (Pereira and Milori, 2010). Recent advances on host-pathogen interactions, genetics of different varieties, and resistance mechanism are discussed in HLB (Duan *et al.*, 2009). The availability of high-

throughput sequencing, transcriptomes and proteomes have been applied to Las-infected plants and treated plants; as a result, numerous characteristic innate immunity elicitors, transcription factors, defense responsive components, and signaling molecules have been discovered (Nwugo *et al.,* 2013b).

Molecular mechanism of *Ca. Liberibacter asiaticus*

The two *Candidatus Liberibacter* species can also be identified by PCR-amplification of their 16S rDNA followed by restriction enzyme (XbaI) analysis of the amplified DNA (Jagoueix *et al.,* 1996). The forward primer OI-1 was defined from the 16S rDNA sequence of *L. asiaticus* and forward primer OA-1, from the 16S rDNA of L. africanum. OA-1 is identical to OI-1 except for three base changes. The reverse primer OI-2c corresponds to identical sequences on the two *Liberibacter* 16S rDNAs. The primer pair OI-1/OI-2c is able to amplify the two *Liberibacter* species; a band of amplified DNA is obtained with as little as 0.1 ng of template DNA. The pair OA-1/OI-2c preferentially amplifies *L. africanum*. In both cases, amplicons close to 1160 bp are obtained. When both *Liberibacter* species are known or suspected to be present, it is recommended to use the two forward primers OI-1 plus OA-1 in the same PCR reaction mixture. Off the shelf DNA extraction kits, including the Sigma RED Extract-N-AmpTM Plant PCR Kit, Sigma, Missouri, USA and the Qiagen DNeasy Plant Mini Kit, can also be used to extract DNA for PCR reactions. The amplicon from the 16S rDNA of *L. asiaticus* contains one Xba1 site and yields 2 fragments (640 bp and 520 bp) upon Xba1 hydrolysis. The amplicon from the 16S rDNA of L. africanum has two Xba1 sites and yields three fragments (520 bp, 500 bp and 130 bp). Thus, Xba1 treatment of the amplicons permits easy distinction between the two Liberibacter species. Details for template DNA preparation (Wizard extracts), PCR detection and identification of two *Liberibacter* species has been published (Jagoueix *et al.,* 1996). Currently, qPCR has become the preferred detection method of *Ca. Liberibacter* spp. (Liu *et al.,* 2011). Compared with conventional PCR, qPCR offers both sensitive and rapid detection of these bacteria. qPCR is reported to increase the sensitivity for *Ca. Liberibacter* spp. detection by 10 times relative to nested PCR and by 100–1000 times relative to conventional PCR for these bacteria (Morgan *et al.,* 2012). The PCR technique can also be applied to the psyllid insect vectors. A PCR method based on the amplification of ribosomal protein genes has been developed. It allows direct distinction between the two *Liberibacter* species based on the difference in size of the amplified DNA (Hocquellet *et al.,* 1999). Two methods for the PCR detection of *L. americanus* have been published (Islam *et al.,* 2012). Monoclonal antibodies are also discovered for the diagnosis of *Ca. Liberibacter asiaticus* and *Ca. Liberibacter africanus* (Garnier *et al.,* 2000). In an alternative method, DNA dot and Southern hybridization was employed by (Hung *et al.,* 2000). In this study, DNA cloning methods were developed and used to detect HLB in infected citrus hosts and the first transcriptome profiling in response to *Ca. Liberibacter* spp. infection using microarray technology was developed by Albrecht and Bowman (2008). 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). 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.

Citrus and Huanglongbing interaction

The molecular interaction between citrus and *Ca. Liberibacter asiaticus* determine whether the citrus will be susceptible, tolerant, or resistant. The host response to HLB pathogen infection of phloem tissue. Despite the many visual and physiological observations on HLB-affected citrus plants worldwide, the molecular determinants for the HLB disease have yet to be established. Phloem is an ideal habitat for more than 12 disease agents including *Phytoplasma* spp., *Spiroplasma* spp. and *Ca. Liberibacter* spp. (Bové, 2006) due to the presence of rich nutrients in phloem sap. Plant defense responses to pathogens are triggered by recognition of conserved pathogen associated molecular patterns (PAMPs) by plant receptors, resulting in a partial reduction of pathogen growth. If the pathogen secretes virulence proteins that are recognized by resistance (R) proteins in the plant, a much stronger form of resistance will occur to arrest pathogen growth completely, making R genes the optimal source of crop disease resistance. Concerted screening efforts have not found evidence of R genes to HLB in any variety of citrus. However, there is substantial variation in the disease tolerance of different citrus varieties and rootstocks tolerance is the ability of a plant to keep growing and producing fruit in the presence of infection. Tolerant citrus varieties have been found to accumulate a greatly reduced pathogen load compared with susceptible varieties (Albrecht and Bowman, 2008) and show less severe symptoms (Fan *et al.,* 2011). Plants use pattern recognition receptors (PRRs), which are typically localized in the plant cell membrane, to respond to microbial- or pathogen-associated typically localized in the plant cell membrane, to respond to microbial- or pathogen-associated molecular patterns (MAMPs or PAMPs, respectively) (Jones and Dangl, 2006). Plants recognize a wide range of bacterial PAMPs, most of which are derived from structural components of the bacterial cell (Cevallos et *al.,* 2012). In addition, *Ca. L. asiaticus* lacks type II plant cell-wall degrading enzymes, which have been known to elicit defense responses based on autodegradation products of the plant cell wall (oligogalacturonides) (Orozco-Cardenas and Ryan, 1999). However, *Ca. L. asiaticus* still contains 57 genes in cell envelope biogenesis, the outer membrane, including lipopolysaccharides (LPS), and most flagellar genes (Duan *et al.,* 2009), which might function as PAMPs. It has been shown that *Ca. L. asiaticus* contains a functional fla gene encoding a flagellin and hook-associated protein of 452 amino acids that contains the conserved flg22 (Zhang *et al.,* 2011). The fla gene could partially complement the corresponding Sinorhizobium meliloti fla mutant. Transient expression in planta indicated that FlaLas induced cell death and callose deposition in *Nicotiana benthamiana* and that the transcription of BAK1 and

SGT1, which are associated with plant innate immunity, was upregulated. The synthetic Flg22L as peptide could not induce plant cell death but retained the ability to induce callose deposition (Zhang *et al.,* 2011). The influence of flagellin and Flg22Las on the induction of cell death and callose deposition is similar to that of other known flagellin and Flg22 (Naito *et al.,* 2008). Thus, it has been suggested that *Ca. L. asiaticus* flagellin may act as a PAMP and trigger host plant resistance to the HLB bacteria (Zhang *et al.,* 2011). However, flagella have not been observed for *Ca. L. asiaticus*, even though most flagellar genes are present in the genome (Duan *et al.,* 2009). In addition, FLAGELLIN- SENSING2 (FLS2) is a transmembrane receptor kinase that binds to bacterial flagellin or flg22 through a physical interaction within the FLS2 extracellular domain (Ali and Reddy, 2008, Dunning *et al.,* 2007). It is unknown how *Ca. L. asiaticus* perceives the flagellin or flg22 and other PAMPs because *Ca. L. asiaticus* resides in the phloem, an intracellular environment rather than an extracellular environment.

Interaction between effector proteins and *Ca. Liberibacter* in citrus

Citrus-*Ca. Liberibacter* interactions also involve effector proteins of bacterial origin that are predicted to modulate host cellular functions for the benefit of *Ca. Liberibacter* multiplication and colonization of host phloem cells (Wang *et al.,* 2006). Generally, microbial effectors play an important role in bacterial pathogenesis by restricting the host defense or interfering with host developmental processes in ways that benefit the pathogen (Jones and Dangl, 2006). *Ca. Liberibacter* genome sequences revealed the presence of substrate proteins derived from type I secretion systems (T1SS)9 and general secretory systems (Sec) in some *Ca. Liberibacters*, but genes for other secretion systems commonly found in other plant-pathogenic bacteria are absent from the *Ca.* Liberibacter genomes sequenced to date (Duan *et al.,* 2009; Leonard *et al.,* 2012). T1SS effector genes encoding serralysin and hemolysin have been identified from CLas isolates, but their role in host interactions remains to be experimentally determined (Duan *et al.,* 2009). More than a hundred genes encoding substrates associated with Sec (containing a signal peptide for the Sec system) have been predicted for CLas strains (Prasad *et al.,* 2016). Some predicted Sec-dependent effectors (SDEs) have been confirmed experimentally as substrates of Sec (Cong et *al.,* 2012; Pitino *et al.,* 2016). Although the role of SDEs in relationship to HLB remains unclear, there is evidence that some could play an important virulence role. For example, Las 5315 has been shown to be localized in the chloroplast and to induce callose deposition and trigger host cell death when expressed transiently in *N. benthamiana* (Pitino *et al.,* 2016). Their role could be deciphered by *in planta* transient or transgenic expression in a plant, perhaps *N. benthamiana* or citrus. The 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 (Chisholm *et al.,* 2006). To simplify the articulation of this concept, any citrus responses that meet these criteria will be referred to as a potential ETI. 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 (Liu *et al.,* 2011). 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.

Transcriptomic responses of citrus plants to *Ca. Liberibacter*

Most importantly, the host plant responds to bacterial infection also at the transcriptional level. Citrus or citrus relatives, whether tolerant or susceptible to HLB, reprogram their transcriptomic network in ones, and stronger in tolerant cultivars than susceptible ones (Rawat *et al.,* 2015). The transcriptomic responses involve upregulated genes that can be categorized into a range of biological processes with which they are associated, such as those related to defense networks, photosynthesis and metabolism, hormone-mediate signaling networks, cell wall metabolism, and reduction/oxidation processes. Most defense/stress response genes are upregulated in all genotypes of host plants at an early stage of infection (Martinelli *et al.,* 2012), but some, such as those involved in the mitogen activated protein kinase (MAPK) signaling pathway, activation of peroxidases, Cu/Zn-superoxide dismutase (Cu/Zn-SOD) and POD4, and nucleotide binding site-leucine rich repeat (NBS-LRR) type genes, are differentially upregulated in tolerant genotypes (Nwugo *et al.,* 2013b). Certain genes reflecting the transcriptomic responses of host plants to *Ca. Liberibacter* infection can be confirmed at the proteomic level. For example, defense-related chitinase, miraculin-like proteins, Cu/Zn superoxide dismutase and lipoxygenase are upregulated in CLas-infected sweet orange plant leaves (Fan *et al.,* 2011). Evidence of up regulation of radical ion detoxification proteins (*e.g.,* glutathione-Stransferases) for tolerance to CLas also has been reported (Martinelli *et al.,* 2012).

Citrus metabolic defense against HLB

Ca. L. asiaticus is parasitic rather than pathogenic, causing host metabolic imbalances by nutrient depletion or interference with transportation, which results in HLB symptoms (Duan *et al.,* 2009). Knowledge of the carbon source and sugar metabolism of the *Ca. L. asiaticus* facilitates understanding of its pathogenicity. *Ca. L. asiaticus* may disrupt host cellular metabolic functions by importing multiple host-cell metabolites for growth and development, ultimately leading to disease expression. *Ca. L. asiaticus* has the ability to metabolize sugars such as

glucose, fructose, and xylulose but not mannose, galactose, rhamnose, or cellulose (Duan *et al.*, 2009). The concentrations of fructose and glucose are very low in the phloem sap (Flowers and Yeo, 1992). Therefore, consumption of fructose by *Ca. L. asiaticus* during infection may initiate a shift in the host metabolite distribution. (Fan *et al.*, 2010) observed a remarkable accumulation of glucose but not fructose and suggested that *Ca. L. asiaticus* might preferentially utilize fructose, similar to *Spiroplasma citri.* Thus, *Ca. L. asiaticus* infection will result in reduced fructose concentrations and the accumulation of glucose in the infected host tissues. Glucose accumulation will subsequently favor the repression of enzymes involved in photosynthesis and contribute to HLB symptom development. Interestingly, the consumption of fructose by *Spiroplasma citri* has been implicated in affecting phloem loading of sucrose, sugar accumulation in source leaves, and causing disease symptoms, including yellowing.

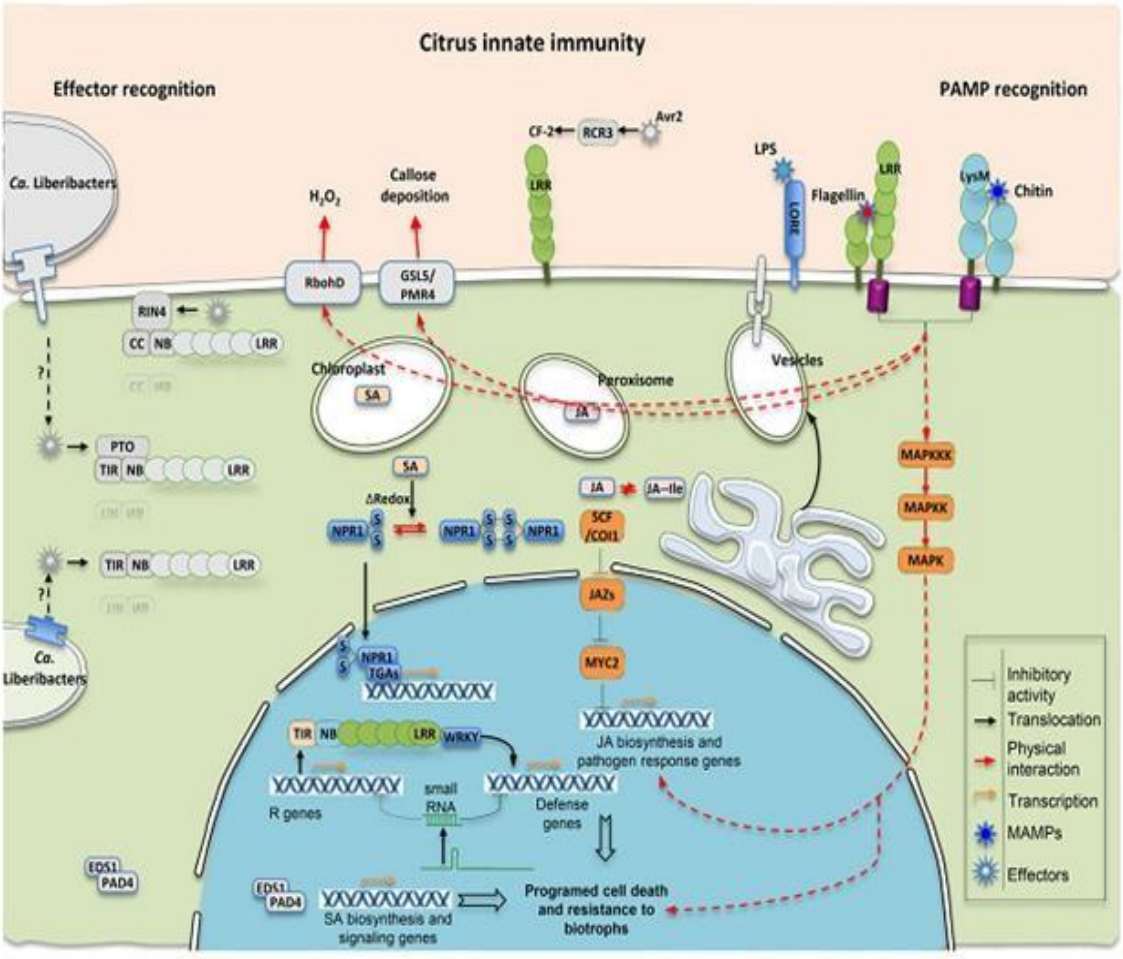

FIGURE 1: The up-to-date identified citrus immunity components (and their associated signaling, catalytic, and metabolic pathways) are projected to an overview of the understanding of plant innate immunity, adapted from (Panstruga *et al.*, 2009) with modification. Reported citrus homologs of innate immunity component 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.

Sugar and starch accumulations have been observed previously in citrus trees infected by *Ca. Liberibacter* (Kim *et al.*, 2009). It is possible that *Ca. L. asiaticus* could affect the phloem loading of sucrose in citrus and result in starch accumulation. Such mechanisms of pathogenicity are based not on specific genes, such as genes for toxins, but on deviations in sugar metabolism. However, *Ca. L. asiaticus* encodes only one sugar transporter for glucose/galactose (Duan *et al.*, 2009). It is unknown how *Ca. L. asiaticus* imports fructose from its host. Thus, this hypothesis needs further validation. *Ca. L. asiaticus* encodes a relatively low number of genes involved in the biosynthesis of compounds, which are readily taken up from the host. Analysis of the de novo amino acid biosynthetic pathways of *Ca. L. asiaticus* has revealed that they are capable of producing serine, glycine, cysteine, aspartate, lysine, threonine, glutamate and arginine and incapable of making histidine, tyrosine, thiamine, phenylalanine, tryptophan, asparagine, isoleucine, methionine, alanine, valine, leucine and proline. Finally, host-pathogen interactions in HLB also include host metabolic responses that may result from the manipulation

of metabolic pathways by the pathogen for its benefit, or may result from host cellular function for defense reaction. One of the most obvious host responses to *Ca. Liberibacter* infection is an increase in total amino acid abundance, indicating benefits to both pathogen and host defense (Killiny and Hijaz, 2016). Other metabolites such as terpenoids, appearing at higher levels in tolerant than in susceptible genotypes, may play an important antibacterial role, restricting pathogen growth, while other metabolites, present at lower levels, may restrict pathogen nutrient acquisition (Killiny and Nehela, 2017).

CONCLUSIONS AND FUTURE PROSPECTS

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. Moreover, latest technologies like chromosome engineering, RNAi, marker-assisted recurrent selection (MARS) and genome-wide selection (GWS), targeted gene replacement using zinc-finger nucleases, next generation sequencing (NGS), Nano biotechnology and genome editing (CRISPR/Cas9) the future seems to more fruitful for the development of citrus with resistance features against the HLB. Thus, interdisciplinary approaches are very important to tackle the HLB.

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