

CITRUS HUANGLONGBING DIAGNOSIS BASED ON MOLECULAR DETECTION OF ASSOCIATED LIBERIBACTER SPECIES

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Abstract

Citrus huanglongbing (HLB) is one of the most devastating citrus diseases in the world. The disease is associated with three species of *Candidatus* Liberibacter, 'Ca. L. asiaticus', 'Ca. L. africanus', and 'Ca. L. americanus', and transmitted mainly by the Asian citrus psyllid (*Diaphorina citri*) and the African citrus psyllid (*Trioza erytreae*). Upon the first report of the disease in São Paulo, Brazil in 2004 and Florida, USA in 2005, HLB started to devastate the two largest citrus industries in the world. The disease continues to rapidly spread to the remaining citrus producing areas still free of the disease, such as Central America and Middle East, besides its presence in almost all Asian citrus producing nations. Early detection of the associated bacterium in host plants and vector insects is essential to HLB control and management. Sampling, sample processing and testing are three major processes in disease detection. The main objective of this invited talk is to compile recent advances in method development of sampling, sample processing and molecular testing of *Candidatus* Liberibacter species associated with the disease, and to make recommendations to increase the efficiency and ability to detect the associated bacterium in survey samples. Based on uneven distribution of the bacterium in plant tissues associated with irregular vector transmission, low bacterial titer during early stages of infection in host plants and vector insects, and recent quantitative data of the bacterial populations in infected plants and vectors, different sampling methods and sample sizes are recommended for host plant and vector insect surveys. These recommendations are useful for surveys in areas with HLB and HLB-like symptomatic, asymptomatic orchards and nurseries. In addition, adequate storage methods are also suggested for plant and insect samples prior to testing for the associated bacterium. Sample processing methods, especially of the bacterial DNA isolation from suspect plant and insect samples were compared. A closed mechanical sample homogenization system with commercial DNA extraction kits using DNA binding filters was highly advised for critical samples to avoid sample cross contamination and to obtain consistently high yield and purity of the bacterial DNA. Positive internal control primer and/or probe sets are proscribed for real-time monitoring on DNA extraction quality and PCR testing efficiency of Liberibacter species from suspect plant and insect samples. In comparison with traditional diagnostic methods including electronic microscopy, ELISA, use of fluorescent substance and starch accumulation in host plants, disease symptomology, and biological indexing, isothermal DNA amplification including the Loop-mediated isothermal amplification (LAMP) and the cyclecleave isothermal and chimeric primer-initiated amplification with probe technology (Cyclecleave ICAN), single and nested conventional PCR, and single and multiplex real-time PCR assays were analyzed for specificity, sensitivity and assay performance. Multiplex quantitative real-time PCR methods using the species-specific primers and/or probes for associated bacterium and the positive internal control primers and/or probes targeting the host plant DNA or vector psyllid DNA were appraised for HLB detection. The potential of 125 selected genes from the complete genome of 'Ca. L. asiaticus' was also discussed for future use in multiple-loci detection.

Introduction

Detection, identification and quantification of plant pathogens are essential to plant pathology. Sampling, sample processing, and testing are three major processes of pathogen detection. Citrus huanglongbing (HLB) is one of the most devastating diseases of citrus worldwide. The disease is associated with phloem-limited infections by any of three species, 'Ca. L. asiaticus', 'Ca. L. africanus' and 'Ca. L. americanus' of uncultured *Liberibacter*. Traditional diagnostic methods have been listed for the disease in previous HLB reviews (Da Graça and Korsten, 2004; Halbert and Manjunath, 2004; Bové, 2006; Gottwald et al. 2007; Brilansky and Rogers, 2007; Wang et al., 2009). The main objective of this invited talk is to present recent advances in research work on methods of sampling, sample processing, and molecular detection of *Candidatus Liberibacter* species associated with HLB.

Sampling and Sample Storage

Sampling methods are very essential to detection, identification and quantification of *Liberibacter* species, since its distribution in host plants and vector psyllids could be irregular or at a very low incidence and/or titer. It is relatively easy to sample from suspect plant materials with HLB or HLB-like symptoms found during a field survey. In this case, 1 to 4 branches or twigs with symptomatic leaves or fruits can be collected from each tree. If no symptoms are found on a suspect tree, one one-year old branch with 5 to 10 leaves can be collected on each of four sides of the upper canopy of the tree. If no branches or twigs are available such as in the case of small nursery trees, collect 1 to 12 fully matured leaves from each tree. In some extreme cases without any leaves left on a plant, stem or root bark or fruit can be used. Plant samples can be collected in individual plastic bags. For vector insect samples, collect nymph and adult psyllids as many as possible on HLB symptomatic branches or suspect trees and put them in individual bottles containing 70% of ethanol. Almost all types of plant parts with phloem tissues, such as petioles, midribs and blades of leaves, bark of stems and roots, petals, pistils and stamen of flowers, peduncles, pericarps, central axis, and seeds of fruits (Li et al. 2009a), various instar nymph and adult psyllids (Hung et al. 2004; Manjunath et al. 2007; Li et al. 2008a) can be used for *Liberibacter* detection.

If possible, transport and store all plant and insect sample collected at a low temperature before pathogen detection, identification and quantification. The sample storage life varies among different detection methods. Fresh materials are usually better for all detection methods. Plant materials can be stored for several days or weeks for serological tests, electronic microscopy and biological indexing, for several months until decay for molecular methods (Li et al., 2006a, 2007a, 2008a). For conventional and real-time PCR tests, vector psyllid samples can be stored in 70% ethanol at a low temperature for more than one year (Li et al. 2008a).

Sample Processing

Procedures of sample processing for serological tests and microscopy observation are difficult to be compared and standardized since they vary much among the laboratories. Usually a relatively large size of samples is used for serological tests. A large sample size is often needed but only a very tiny part of the sample is actually used for microscopy observation. Only a limited size of plant samples can be used for DNA

isolation of Liberibacters for molecular assays. Modified and simplified CTAB methods and commercial DNA kits are among the best in terms of DNA yields, quality and amplification efficiency for pathogen isolations from citrus plants (Li et al. 2006b, 2007b) and vector insects (Hung et al. 1999; Manjunath et al. 2007; Li et al. 2008b). A relatively large sample of up to 500 mg of fresh tissues of HLB suspect plants can be used for CTAB methods (Teixeira et al. 2005) and an optimized sample size of 200 mg per extraction was established for the DNeasy Plant Mini kit of Qiagen (Li et al. 2006a, 2007a). Liberibacter DNA can be successfully obtained from one to one hundred nymphs or one to fifty adults of psyllids per extraction by the modified CTAB methods (Hung et al., 2004; Manjunath et al., 2007; Li et al. 2008b) and the DNeasy Blood & Tissue kit of Qiagen (Li et al. 2008b).

The extraction efficiency of the total DNA isolation from suspect samples can easily be quantified by the positive internal control primer-probe set COXfpr for the host plants (Li et al. 2006a) and the positive internal control primer-probe set WGfpr for the vector psyllids (Li et al. 2008b) used in the multiplex real-time PCR assays. The quality of the total DNA extracts can be also quantitatively assessed through the PCR amplification efficiency which is calculated from the slopes of the sample standard curves for quantitative assays of the Liberibacters (Li et al. 2008a).

It is essential to any DNA extraction from plants or insects to obtain a total DNA of a constant quality and yield without cross contamination among samples by a closed sample homogenization system for thoroughly and uniformly grinding the suspect tissues. The closed system of a FastPrep instrument (Biomedicals, Irvine, CA) or a Beadbeater (Biospec Products, Inc., Bartlesville, OK) together with the Bio 101 Matrix A tubes can meet the requirements (Li et al. 2006a, 2008b). The traditional manner of grinding plant or insect samples with a plunger or pestle in liquid nitrogen in a mortar can be used for DNA extraction from suspect plant and insect samples for Liberibacter detection (Jagoueix et al. 1996; Hocquellet et al. 1999; Hung et al. 2004; Teixeira et al. 2005; Manjunath et al. 2007; Tatineni et al. 2008), but may cause uncontrollable variations in DNA quality and quantity and unavoidable cross contaminations between samples.

The variations in DNA quality and quantity and the cross contaminations among samples are usually invisible by most low-sensitive diagnostic methods such as microscopy, ELISA and conventional PCR, but become noticeable by sensitive methods such as real-time PCR (Li et al. 2006a). In addition, it is almost impossible to obtain a constant DNA yield per sample size by traditional DNA isolation through phenol-chloroform precipitation and washing with ethanol. The DNA binding filters as used in Qiagen kits can be used in quantitative DNA extraction methods for quantitative detection of the Liberibacters in plants and psyllids. The quality and the quantity of the total DNA extracts including Liberibacter DNA, host plant or vector insect DNA can be readily evaluated by the multiplex real-time PCR with the Liberibacter-targeted primer/probe sets (Li et al., 2006) and the positive internal control primer/probe sets targeting at the host plant (Li et al., 2006) and vector psyllid (Li et al., 2008b) DNA.

Traditional Diagnostic Methods

There were various traditional detection methods for Liberibacter species. The electronic microscopy (EM) was the only reliable detection method for the bacterium from the first EM observation of a “mycoplasma-like organism” in phloem tissues of HLB-

infected citrus in 1970 (Lafleche & Bove, 1970) to the first DNA probe specific to the bacterium (Villechanoux et al. 1992). However, the EM observation was time-consuming and unable to distinguish species of Liberibacters. A method based on the identification of a fluorescent gentiosyl-glucoside from infected fruits and bark was developed in 1968 (Schwarz 1968a) for confirmation of the disease. However, this method soon proved non-specific since stressed trees contained the same marker (Schwarz, 1970). Monoclonal antibodies were raised for ELISA tests first in 1987 (Garnier et al. 1987), but they were too isolate-specific to be used for detection of different isolates of the bacterium associated with the disease. Two DNA probes were developed specific to 'Ca. L. asiaticus' and 'Ca. L. africanus' (Villechanoux et al. 1992), but the detection sensitivity of the dot-blot hybridization using these probes was similar to that of EM (Jagoueix et al. 1996).

Recently, an iodine reaction (IR) kit was developed (Onuki et al. 2002) based on the elevated starch accumulation in HLB-diseased citrus leaves (Schneider H, 1968). However, the IR method is only a little better than observation of visual symptoms of the disease. Field HLB diagnosis based on symptoms is usually difficult because none of the symptoms is specific (Bové, 2006). The biological indexing by plant indicators such as sweet orange (Schwarz 1968b) and Ponkan mandarin (Matsumoto et al. 1968) was another confirmatory test of HLB. However, the transmission percentages varied from 54.7 to 88.0% for 'Ca. L. asiaticus' and 10.0 to 45.2% for 'Ca. L. americanus' even by the very efficient "seedling inoculation method" for citrus diseases (Li et al., 1996) with a large budstick up to 4 cm in length from HLB symptomatic twigs (Lopes et al., 2009).

Conventional PCR

Since its first introduction by Kerry Mullis in 1983 (Mullis, 1990) for which he won the Nobel Prize in 1993, PCR has become a powerful technique for detection and identification of plant pathogens. The first sets of 16S rDNA-based primers (OI1/OI2c and OA1/OI2c) were designed specific for conventional PCR to detect 'Ca. L. asiaticus' and 'Ca. L. africanus', yielding the same size of 1160 bp of 16S rDNA fragments (Jagoueix et al. 1996). A time-consuming enzyme digestion of the 1160 bp PCR products with *Xba*I is needed to distinguish the two Liberibacter species. In China, one specific primer set was developed based for detection of 'Ca. L. asiaticus' (Tian et al. 1996). In 1999, the conventional PCR primer set A2/J5 was developed based on the β - operon ribosomal protein gene (Hocquellet et al. 1999). This set of primers allows identification of the two Liberibacter species directly by the PCR amplicon size of 703 bp for 'Ca. L. asiaticus' and 669 bp for 'Ca. L. africanus'. Also in 1999, another primer set was developed specific to 'Ca. L. asiaticus' based on DNA fragments directly obtained from HLB-infected citrus in Taiwan (Hung et al. 1999). This set of primers produces an amplicon of 226 bp only with strains of 'Ca. L. asiaticus' not with strains of 'Ca. L. africanus'. Since none of the primer sets above could detect the new Liberibacter species 'Ca. L. americanus', a new set of 16S rDNA-based primers (GB1/GB3) was developed, producing an amplicon of 1027 bp (Teixiera et al. 2005).

The universal 16S rDNA-based primer set fD1/rD1 (Weisburg et al. 1991) was used in the first round of the nested PCR assays to improve the detection sensitivity of single conventional PCR assays with the 16S rDNA-based primer sets OI1/OI2c for 'Ca. L. asiaticus' (Deng et al. 2007) and GB1/GB3 for 'Ca. L. americanus' (Teixiera et al. 2005). The primer set was also nested with another 16S rDNA-based primer set CGO3F/CGO5R for detection of 'Ca. L. asiaticus' in *Murraya paniculata* (Zhou et al.

2007). The nested conventional PCR was still at list 10 to 100 fold less sensitive than real-time PCR (Teixiera et al., 2008)

The loop-mediated isothermal amplification (LAMP) method was developed for detection of Liberibacters in under-equipped laboratories without a thermal cycler (Okuda et al. 2005). Although the LAMP method has a similar sensitivity to conventional PCR assays, it is more vulnerable to contaminations than the later (Li et al. 2007a). In 2008, LAMP was developed into a cycleave isothermal and chimeric primer-initiated amplification of nucleic acids with probe technology (Cycleave ICAN) (Urasaki et al., 2008). Cycleave ICAN method is a little more sensitive than conventional PCR, and can be used for detection of the Liberibacters in screening laboratories without a thermocycler.

Real-time PCR

Since the Applied Biosystems (ABI) made the first real-time instrument 7700 commercially available in 1996 (Heid et al. 1996), real-time quantitative PCR (qPCR) has become the most accurate and sensitive method for the detection and quantification of nucleic acids yet devised (Shiple 2006). The first TaqMan probe of real-time qPCR for Liberibacter detection was designed in 2004 (Liao et al. 2004) based on the 16S rDNA fragment amplified by the conventional PCR primer set OI1/OI2c (Jagoueix et al. 1996) from a HLB-infected citrus plant in Fujian, China. However, its amplicon by was 441 bp which was out of the amplicon size range (50-250 bp) for real-time PCR (Wang and Seed, 2006). This too long amplicon leads to decreased PCR efficiency and detection sensitivity with a low detection limit of up 1,000 copies of templates of cloned plasmid DNA per reaction.

In 2005, species-specific TaqMan probe-primer sets, HLBaspr, HLBafpr and HLBampr were developed for detection and identification of the three known species of Liberibacter in complex PCR with the positive internal control TaqMan probe-primer set COXfpr targeting the host plant cytochrome oxidase gene (Li et al. 2006a). The low detection limits of the three HLB probe-primer sets are down to 1 to 10 copies of Liberibacter's 16S rDNA per reaction and their PCR efficiency is up to 99.90%. In addition, the low detection limit and the PCR efficiency of the positive internal control probe-primer set COXfpr are almost the same as those of the HLB probe-primer sets, which allows accurate estimation of the ratio of the Liberibacter DNA to the host plant DNA in total DNA extracts obtained from infected plants. The HLBaspr set has been successfully applied in detection, identification and quantification of 'Ca. *L. asiaticus*' in host plants of citrus (Tatineni et al. 2008; Li et al. 2008c) and in vector psyllids (Manjunath et al. 2007).

Based on a β -operon protein gene of a DNA isolate obtained from HLB-infected citrus in Quangxi, China in 2006, one TaqMan probe-primer set CQULA04f/r/p10 was developed specifically for detection and quantification of 'Ca. *L. asiaticus*' (Wang et al., 2006). This primer pair was also employed in the SYBR Green real-time PCR (Wang et al. 2006). In 2008, another β -operon-based primer set was developed in Brazil for SYBR Green real-time PCR to study the distribution and quantification of 'Ca. *Liberibacter americanus*' in citrus plants (Teixeira et al. 2008). The PCR efficiency and sensitivity of the two β -operon-based qPCR primer and/or probe sets were similar to those of the 16S rDNA-based TaqMan probe-primer sets HLBaspr and HLBampr (Li et al. 2006a, 2007a). However, the former two sets produce real-time PCR results of Ct values about 2 cycles

higher than the later two sets. This is due to that there are three operons of 16S rRNA per genome of the bacterium while only one copy of β -operon per genome. So, the detection sensitivity of the 16S rDNA-based real-time PCR is a little higher than that of the β -operon-based one in assay performance for real plant and insect samples. In addition, the real-time PCR assays are at least 10 to 100 fold more sensitive than the conventional PCR methods for detection of *Liberibacter* species associated with HLB (Li et al., 2006; Wang et al., 2006; Teixeira et al., 2008).

A very sensitive and stable positive internal control TaqMan probe-primer set WGfpr was successfully developed in 2007 for real-time qPCR multiplexed with HLBaspr or HLBafpr or HLBamp for detection, identification and quantification of the three unknown species of *Liberibacter* in vector psyllids (Li et al. 2008b). The quality of DNA extraction from *Liberibacter*-suspect citrus psyllids can be evaluated by the positive internal control. '*Ca. L. asiaticus*' was readily detected and quantified in a single infected nymph or adult psyllid even if it was in a sample composed with other 100 *Liberibacter*-free nymphs or adults (Li et al. 2009c, in submission). The discovery of the elevated ratios of '*Ca. L. asiaticus*' DNA to the Asian citrus psyllid DNA by the multiplex real-time PCR has been successfully used in the *Liberibacter* genome sequencing project (Duan et al., 2009).

Multiple Genetic Loci for Detection

Only few genetic loci of the *Liberibacter* species were used for diagnosis of the associated citrus disease, since limited gene sequences of the bacterium were available due to the difficulty of its isolation and cultivation (Davis et al., 2008; Sechler et al., 2009). Although there are nine domains in the bacterial 16S rRNA gene (Baker et al., 2003; Sontakke et al., 2009), the 16S rDNA PCR assays using species-specific primers and/or probes (Jagoueix et al., 1996; eixeira et al., 2005; Li et al., 2006a) are to date the most popular and the most confidential method for detection of *Liberibacter* species associated with HLB. The conserved 16S rRNA can be used as a housekeeping gene for detection of *Liberibacter* species if primers and/or probes are designed on its species signature (domain-specific) region (Jagoueix et al., 1996; Teixeira et al., 2005; Li et al., 2006a). These species-specific primers and/or probes can detect all isolates of each of the three species of *Liberibacter* associated with HLB and do not react with the new *Liberibacter* species, '*Ca. L. solanacearum*' associated with zebra chip disease of potato (Li et al., 2009b), neither other bacteria causing false positive results. However, 16S rDNA-based but domain-beyond primers and/or primers could yield false positive detection results.

Sequences of several other non-conserved genes, such as β -operon protein gene (Hocquellet et al., 1999; Wang et al., 2006, Teixeira et al., 2008), outer membrane protein gene (Bastianel et al., 2005), and DNA polymerase gene (Tomimura et al., 2009) were used for HLB diagnosis. However, primers and/or probes based on these sequences could yield false negative and/or false positive results (Tomimura et al., 2009). There are 1,134 ORFs (genes) in the complete genome of '*Ca. L. asiaticus*' (Duan et al., 2009). To validate alternative candidate genes for diagnostic purposes, we have designed primers and TaqMan probes for each of 125 ORFs selected from the whole genome. We are in the progress of an extensive national and international collaboration to screen these primers and probes against *Liberibacter* DNA isolates from all HLB positive counties of Florida, Louisiana, South Carolina and Georgia, and from many other HLB-positive citrus producing nations.

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