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A new diagnostic system for ultra-sensitive and specific detection and quantification of *Candidatus* Liberibacter asiaticus, the bacterium associated with citrus Huanglongbing

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ABSTRACT

An ultra-sensitive and quantitative diagnostic system by combining nested PCR and TaqMan® PCR in a single tube was developed for detection of "*Candidatus* Liberibacter asiaticus". The procedure involves two PCR steps using the species-specific outer and inner primer pairs. Different annealing temperatures allow both the first and the second rounds of PCR to be performed sequentially in the same closed tube. The first PCR with outer primers was performed at a higher annealing temperature and with limited amount of primers to prevent interference with the inner primers during the second round of PCR. The specificity of the dual primer TaqMan® is high because the fluorescent signal can only be generated from the TaqMan® probes that are homologous to the product amplified by the outer and inner primers. This new detection system can reliably detect as few as single copies of target DNA. The sensitivity of the dual primer system is comparable to the conventional two-tube nested PCR, but it eliminates the potential risk of cross contamination commonly associated with conventional nested PCR. This one-tube dual primer TaqMan® PCR method is gel-free with reduced handling time and is cost effective. At the same time, this system provides significantly increased sensitivity, improved reliability and high through-put capability suitable for routine, large scale diagnoses of clinical plant tissue and insect samples. The technique described here is generic and can be applied to the detection of other plant pathogenic bacteria.

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1. Introduction

Citrus Huanglongbing (HLB, also known as greening disease) is one of the most devastating diseases that threaten citrus production worldwide (Bové, 2006; Halbert and Manjunath, 2004). Three "*Candidatus* Liberibacter" species, "*Candidatus* Liberibacter asiaticus" (Las), "*Ca.* L. africanus" (Laf) and "*Ca.* L. americanus" (Lam), are associated with various forms of HLB disease (Bové, 2006; Bové et al., 1974; Gottwald et al., 2007). The Gram-negative bacterium is phloem-limited and belongs to the α subdivision of the *Proteobacteria*. These HLB-associated prokaryotes can be transmitted by dodder as well as by budding or grafting with propagative material from HLB-affected plants. Under natural conditions, the HLB-associated Liberibacter species are transmitted by the psyllid vectors, *Diaphorina citri* (Kuwayama) in the Asia and America (Halbert and Manjunath, 2004), and *Trioza erytreae* (Del Guercio) (Homoptera: Psyllidae) in Africa (Bové, 2006; Catling, 1969; McClean, 1974; McClean and Oberholzer, 1965). Among the three HLB-associated Liberibacter species, Las is the most widespread and is responsible for increasing economic losses worldwide in the recent years. The disease has been known in Asian countries since the 1870s (Beattie et al., 2008; Bové, 2006; Lin, 1956) but has been emerging as a serious economic threat to citrus production in the western hemisphere in recent years (Bové, 2006; Gottwald et al., 2007). HLB, associated with Las, was recently reported in São Paulo State (Brazil) (Coletta-Filho et al., 2004; Teixeira et al., 2005), Florida (USA) (Halbert, 2005), Cuba in 2006 (Martínez et al., 2008) and subsequently in the Dominican Republic in 2008 (Matos et al., 2009). More recently, the disease was also confirmed to be present in the states of Yucatán, Nayarit and Jalisco, Mexico in 2009 (NAPPO, 2009).

Studies of HLB have been impeded because Koch's postulates *sensu stricto* have not been conclusively fulfilled (Sechler et al., 2009). Early disease detection relies primarily on scouting for disease symptoms in the field, such as yellow shoots, blotchy mottle leaf and lopsided fruit with green color remaining at the stylar end and aborted seeds (Bové, 2006; Gottwald et al., 2007). However, HLB diagnosis based on disease

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symptoms can be difficult because they may be complicated by other biotic and/or abiotic plant health related problems (Bové, 2006). While diagnostic methods, such as microscopy, serology and DNA–DNA hybridization, have been used for detection purposes (Varma et al., 1993; Villechanoux et al., 1990), these methods are either time consuming or lack appropriate sensitivity. PCR-based molecular diagnostic methods, which are rapid, sensitive, specific and reliable, have been widely used for clinical diagnosis of HLB-associated Liberibacters (Hocquellet et al., 1999; Hung et al., 1999; Kunimasa et al., 2006; Li et al., 2006; Okuda et al., 2008; Teixeira et al., 2008; Villechanoux et al., 1993, 1992). These methods have also been used for Liberibacter detection in psyllid vectors (Manjunath et al., 2008). However, application of these methods for HLB detection, particularly for early detection, is inconsistent due to low bacterial titers in citrus and uneven distribution of Las in symptomatic and non-symptomatic field-grown trees (Li et al., 2006; Manjunath et al., 2008). The sensitivity of detection of HLB-associated Liberibacters, therefore, has been improved by adapting a nested PCR procedure (Deng et al., 2007; Ding et al., 2004; Li and Ke, 2002). Introduction of a second pair of primers, resulting in amplification of the previously amplified material, could lead to a significant increase in sensitivity of detection. Nested PCR has been used to improve the sensitivity of HLB-associated Liberibacter detection in clinically asymptomatic trees, especially in asymptomatic nursery plants, citrus relatives with low HLB-associated Liberibacter titer and for possible seed transmission (Benyon et al., 2008; Ding et al., 2004). However, the procedure is time consuming, as it involves two sequential rounds of PCR. The operational cost of nested PCR is nearly twice that of standard PCR. More importantly, the introduction of first round PCR amplification products to another tube for the second round of PCR amplification could potentially lead to significant percentage of false positives due to cross-contamination rendering this approach too risky for practical applications (Llop et al., 2000).

To maintain high sensitivity while overcoming these drawbacks, we designed and developed an ultra-sensitive "single closed tube dual primer" (STDP) TaqMan® PCR system. In contrast to conventional two-tube nested PCR, this detection method is based on two sequential PCR amplifications in a single closed tube, thereby eliminating the potential risk of cross-contaminations commonly associated with conventional two-tube nested PCR. The new system is a TaqMan®-based quantitative PCR which is gel-free, and easier to perform than two-tube nested PCR. The only extra cost is for the outer primers compared to standard TaqMan® PCR. The assay system provides ultra-sensitivity and reliability for HLB-associated Liberibacter detection. The system is useful for year-around disease or HLB-associated Liberibacter surveys and epidemiological studies. The assay system is also suitable for a large scale sample screening in HLB-affected areas, as well as in areas where HLB is a potential threat, but has not yet been found.

2. Materials and methods

2.1. Sample sources and DNA preparation

Leaf samples from 250 citrus trees with variable blotchy mottle symptom severity were collected from 11 citrus orchards and from 20 1–2 year symptomless nursery trees in Nanning City, China. In addition, leaf samples were also collected from 20 *Murraya paniculata* (orange jasmine) and 20 *Clausena lansium* (Chinese wampee) in Guilin City, China, respectively. Leaves were washed under running tap water and blotted dry with paper towels. The midribs were then excised from the leaf blade. Total genomic DNA was extracted from 4–5 midribs per sample. Samples were ground in liquid nitrogen and DNA was extracted using the CTAB method as previously described (Lin et al., 2008). Precipitated DNA was dissolved in 100 µl of TE buffer. The quality of DNA samples was checked by electrophoresis in 1.2% agarose gels. DNA concentrations were determined spectrophotometrically and adjusted to 50 ng/µl. Due to the select agent status of the "*Candidatus* Liberibacter" species at that time, HLB-infected DNA samples from São Paulo, Brazil, India, Florida and China were extracted in their respective origins and sent to California as microbially-sterile, non-infectious DNA samples. HLB-associated Liberibacter-free DNA samples used as negative controls were prepared from citrus leaves collected in an orchard at the USDA-Agricultural Research Service, San Joaquin Valley Agricultural Sciences Center in Parlier, CA.

2.2. Primer and probe designs

"*Ca.* Liberibacter asiaticus" sequences representing a single copy in the Las genome were selected (Duan et al., 2009) for designing TaqMan® primers and probe for STDP TaqMan® PCR. This region has 8371 bp, of which 3701 bp were previously reported in a region containing the genes, "outer membrane protein and UDP-3-O-3-hydroxymyristoyl glucosamine N-acyltransferase" (accession # AY642159) (Bastianel et al., 2005) and 4150 bp were obtained using the genomic walking method (accession # EU523377 and EF164804) containing the genes, "elongation factor Ts", "uridylate kinase", "ribosome recycling factor", and "undecaprenyl diphosphate synthase" (Lin et al., 2008; Doddapaneni et al., 2008). The optimum TaqMan® probe and inner primers were designed using Beacon Design Software v7.0 (Premier Biosoft International, CA, USA) with the following criteria: GC % ≥ 40–50, Tm = 55 °C ± 2, primer length = 18–22 bp with amplicon size ranging from 120 to 200 bp. The melting temperature (Tm) for the TaqMan® probe was set 10 °C higher than the Tm for inner primer. To ensure amplification efficiency, the primers and probes were designed in a region where no secondary structures have been observed. Among the designed primers and probes, only those having the least possibility of forming a hairpin, self/cross dimer structures were selected for further validation. For designing the outer primers, the same criteria were applied, except that a higher Tm of 65 °C (10 °C higher than inner primer Tm) and longer amplicon size (i.e., 300–500 bp) flanking upstream and downstream of forward and reverse inner primers were selected. A computational algorithm was then performed to conduct pair-wise comparisons of all primer/primer and primer/probe and to select the best primers/probe set combination that had the least stability of forming self/cross dimers between inner and outer primers and between primer and probe ($\Delta G \geq -2$ kcal/mol). These sequence analyses resulted in identification of three sets of inner/outer primers and TaqMan® probes. The fluorescent reporter dye, 6-carboxyl-fluorescein (FAM) was labeled at the 5′ end of the TaqMan® probe. A non-fluorescent quencher, minor groove binder (MGB) was labeled on the 3′ end of probe. The probe was synthesized by Applied Biosystems Inc. (ABI, Foster City, CA).

2.3. Validation of primer and probe specificity

To ensure that the designed primers and probes were unique to "*Ca.* Liberibacter asiaticus", an *in silico* search was performed against available microbial sequences in GenBank. The specificities of the inner and outer primers were also PCR-evaluated with DNA prepared from a number of citrus-related pathogens, including *Xanthomonas citri* subsp. *citri*, the causal agent of citrus canker; *Xylella fastidiosa* strain 9a5c, the causal agent of citrus variegated chlorosis; and *Spiroplasma citri*, the causal agent of citrus stubborn disease. Further, Laf, Lam which are other citrus HLB-associated Liberibacter species, and "*Ca.* L. solanaceraum" that is associated with solanaceous plant diseases, were also included for specificity evaluation.

2.4. Optimizations of PCR conditions

For an efficacious, reliable single closed tube dual primer PCR, the annealing temperatures for inner and outer primers need to be optimized. The first PCR round should be performed at a higher