3.3 Detection of "Candidatus Liberibacter asiaticus" by cycleave isothermal and chimeric primer-initiated amplification of nucleic acids (Cycleave ICAN)

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In Japan, Huanglongbing (HLB) caused by “Ca. L. asiaticus” was first observed in 1988 on Iriomote Island, located about 200 km east of Taiwan (Miyakawa and Tsuno 1989). Subsequently, the widespread occurrence of HLB on Okinawa prefecture was reported (Toguchi and Kawano 1997). HLB is managed by the propagation of clean nursery stock and healthy trees (Gonzales and Su 1988) and the control of the vector, citrus psyllid (Diaphorina citri). Furthermore, trees in commercial citrus groves and private yards need to be tested regularly by polymerase chain reaction (PCR), so that diseased trees can be removed to prevent the spread of HLB. Currently, the immediate removal of the diseased trees is of primary importance for managing HLB (Takushi et al. 2007). In the testing of citrus trees and psyllids, the PCR detection system involved the amplification of the 16S rDNA from “Ca. L. asiaticus” (Jagoueix et al. 1996) and agarose gel electrophoresis. But this system requires too much time to test hundreds of samples; consequently, diseased trees cannot be removed quickly. A rapid, sensitive method that does not require highly advanced equipment such as a high-throughput thermal cycler, is thus needed for the practical detection of “Ca. L. asiaticus”. A recently developed technique, isothermal and chimeric primer-initiated amplification of nucleic acids (ICAN) (Shimada et al. 2002; Mukai et al. 2007), has shown promise for such rapid, bulk detection and uses BcaBEST DNA polymerase, Tli RNaseH, and two DNA–RNA chimeric primers. ICAN has also been combined with luminescence detection by probe hybridization for detecting the Mycobacterium tuberculosis IS6110 insertion element (Shimada et al. 2002), Salmonella sp. invA gene (Isogai et al. 2005), fluoroquinolone-resistant Neisseria gonorrhoeae (Horii et al. 2006), and for simultaneously detecting Chlamydia trachomatis Cryptic plasmid and Neisseria gonorrhoeae CppB gene (Shimada et al. 2003). ICAN, done in a water bath in the case of the invA gene of Salmonella species, is suitable for a large-scale detection system without additional equipment. Therefore, we have developed a “Ca. L.asiaticus” detection system that comprises the amplification of 16S rDNA by ICAN and the detection of the amplified products with cycling probe technology (Bekkaoui et al. 1996; Esaki et al. 2004) (Cycleave ICAN;Urasaki et al. 2007). Although information on the DNA of “Ca. L. asiaticus”, a nonculturable fastidious bacterium, is limited, the ICAN, which only requires two DNA–RNA chimeric primers, can be carried out using the limited information, i.e. the 16S rDNA sequence. The cycling probe is a chimeric DNA–RNA probe that hybridizes to an amplified target sequence, not to a nonspecific product, primer dimers. Once the probe hybridizes, the RNA part of the probe is cleaved by Tli RNaseH. Thus the fluorescent molecule ROX and quencher molecule Eclipse on each side of the probe are separated, and red fluorescence is emitted. With this cycling probe technology, we can rapidly obtain red fluorescence from “Ca. L. asiaticus”-positive samples, and prevent the occurrence of the false-positives.

The performance of Cycleave ICAN and the conventional PCR system (Urasaki et al. 2007) were compared (Fig. 1) using the PCR primers for “Ca. L. asiaticus” 16S rDNA designed by Jagoueix et al. (1996). For the performance test, the DNAs from the healthy and HLB-diseased
*Citrus depressa* “Shiikuwasha”, maintained in the glasshouse, were used as negative and positive templates, respectively. The PCR system clearly detected a 1,160-bp rDNA fragment in the positive templates from 40 ng to 12.8 pg, but not in the positive templates from 2.56 to 0.102 pg. In the Cycleave ICAN, red fluorescence was detected from 40 ng to 0.512 pg. Weak fluorescence was obtained from 0.102 pg. There was no fluorescence from the healthy sample. From this result, the sensitivity of the Cycleave ICAN is considered to be at least 25 times higher than that of the PCR–agarose gel system. The cycling probe enables the reaction to be done in one tube and to provide rapid results without electrophoresis. Compared with the PCR system, the Cycleave ICAN could shorten the time for the detection of “Ca. L. asiaticus”. In our laboratory tests on 44 samples, Cycleave ICAN saved us 2.25 h (1.25 h for the thermal cycling, 1 h for the electrophoresis). The Cycleave ICAN, with detection of “Ca. L. asiaticus” and reliability equivalent to results from the PCR, is a rapid and sensitive detection method. Furthermore, with the Cycleave ICAN the number of samples can be greatly scaled up without the need to purchase additional equipment. The Cycleave ICAN detection system for “Ca. L. asiaticus” will thus greatly facilitate the timely removal of HLB-diseased trees to prevent the spread of the pathogen.

**Citations**


Fig1 Performance test of the PCR and the Cycleave ICAN detection system for “Candidatus Liberibacter asiaticus”. **M:** 100bp DNA ladder; **N:** 50 ng of DNA from leaf midrib of healthy *Citrus depressa* (Shiikuwasha); DNA from leaf midrib of HLB-diseased Shiikuwasha: 1:40 ng, 2:8 ng, 3:1.6 ng, 4:0.32 ng, 5:64 pg, 7:2.56 pg, 8:0.512 pg, 9:0.102 pg