Journal of Integrative Agriculture 2018, 17(2): 483–487

Available online at www.sciencedirect.com

ScienceDirect

SHORT COMMUNICATION

Development of a sensitive and reliable droplet digital PCR assay for the detection of '*Candidatus* Liberibacter asiaticus'

ZHONG Xi[1], LIU Xue-lu[1], LOU Bing-hai[2], ZHOU Chang-yong[1], WANG Xue-feng[1]

[1] *National Citrus Engineering Research Center, Citrus Research Institute, Chinese Academy of Agricultural Sciences/Southwest University, Chongqing 400712, P.R.China*

[2] *Guangxi Key Laboratory of Citrus Biology, Guangxi Academy of Specialty Crops, Guilin 541004, P.R.China*

Abstract

Citrus Huanglongbing (HLB, yellow shoot disease) is one of the most serious citrus diseases worldwide. To better improve the detection sensitivity, a droplet digital PCR (ddPCR) assay was developed for the rapid detection of '*Candidatus* Liberibacter asiaticus' (Las), the putative causal agent of HLB. The detection of sensitivity comparison using positive plasmid indicated that ddPCR was superior to quantitative PCR (qPCR) for detecting and quantifying Las at low concentrations. The Las detection of 40 field samples also showed that six of 13 asymptomatic samples (46.15%) with high C_t value (>35) were positive by ddPCR. This methodology showed great potential for early HLB infection diagnosis.

Keywords: citrus Huanglongbing, early diagnosis, droplet digital PCR

1. Introduction

'*Candidatus* Liberibacter asiaticus' (Las), a phloem-resided α-protebacterium, is the putative causal agent of citrus Huanglongbing (HLB, yellow shoot disease) that is one of the most serious diseases in citrus production (Bové 2006). The bacterium is transmitted from infected to healthy plants through grafting or by citrus psyllid (*Diaphorina citri*). No effective cure is currently available for HLB-infected citrus plants. Therefore, the use of pathogen-free nursery stocks, control of insect vector and removal of infected trees are major control measures in HLB management. This is of particular importance if HLB infection status of asymptomatic trees in field could be accurately diagnosed for the implementation of control strategies.

Because Las is unable to be cultured so far, current detection is typically PCR-based using primers developed from genomic DNA sequences, mostly the 16S rRNA gene. Primer set OI1/OI2c for conventional PCR and primer-probe set HLBas/HLBp/HLBr for TaqMan real-time quantitative PCR (qPCR) are widely used for the standardized detection of Las (Jagoueix *et al*. 1994; Li *et al*. 2006). Recently, multi-copy genes have been chosen as targets for the improvement of qPCR sensitivity (Morgan *et al*. 2012; Zheng *et al*. 2016). However, absolute quantification of unculturable Las by qPCR is challenging due to erratic distribution and low titer, especially for early detection of Las infection.

Droplet digital PCR (ddPCR) is a new technology that allows sensitive detection and absolute quantification of low concentration DNA without the need for a standard curve.

Received 10 July, 2017 Accepted 31 October, 2017
ZHONG Xi, E-mail: zhongmxi@yeah.net; Correspongdence WANG Xue-feng, Tel: +86-23-68349338; E-mail: wangxuefeng@cric.cn

doi: 10.1016/S2095-3119(17)61815-X

Each sample tested was partitioned in tens of thousands of individual droplets in a water-oil emulsion and then the number of positive droplets was read by cumulative fluorescence signal during PCR amplification. The total number of target DNA molecules in a sample can be calculated from the fraction of positive droplets and Poisson statistics (Hindson *et al*. 2011). Since ddPCR has been shown to yield more precise detection results than qPCR, the robust and powerful method has been increasingly used in medical researches (Taylor *et al*. 2015), clinical applications (Tsui *et al*. 2011; Watanabe *et al*. 2015), food safety inspection (Pinheiro *et al*. 2011; Floren *et al*. 2015) and gene-editing frequencies study (Mock *et al*. 2016). Recently, it also has been used to detect *Xanthomonas citri* subsp. *citri*, an economically important disease of citrus (Zhao *et al*. 2016).

In this study, we established ddPCR approach to detect and quantify Las in both symptomatic and asymptomatic samples. The detection sensitivity of ddPCR was compared to qPCR targeting the gene encoding 16S rRNA.

2. Materials and methods

2.1. Sample collection and DNA extraction

HLB symptomatic and asymptomatic field citrus samples were collected from Guangxi and Hunan of China. All collected samples in China were shipped by mail to Citrus Research Institute (CRI) of Southwest University in Chongqing, China. Four HLB-positive citrus samples and four negative citrus samples were collected from the greenhouse in CRI. The midribs of citrus leaves were excised and DNA was extracted using the cetyltrimethylammonium bromide (CTAB) methods as previously described (Wang *et al*. 2012).

2.2. Preparation of cloned plasmid standard

A DNA segment encoding 16S rRNA gene of Las was amplified with Las genomic DNA as the template. The PCR amplicon was purified and ligated into the pEASY-T1 cloning vector (TransGen Biotech, China). Plasmid DNA was extracted from transformed competent cells and used to generate a standard curve for tenfold serial dilutions consisting of nine concentration gradients, which were used to test the sensitivities and linearity range of qPCR and ddPCR assays.

2.3. Quantitative PCR

The primers and probe targeted the 16S rRNA gene of Las were used in the subsequent qPCR and ddPCR assays (Li *et al*. 2006). The qPCR assay was performed on an iCyler IQ™ System (Bio-Rad, Hercules, CA, USA). The cycling conditions included incubation for 30 s at 95°C followed by 40 cycles of 95°C for 5 s and 58°C for 30 s. C_t values were analyzed using BioRad iCycler iQ version 3.0 Software with auto-calculated baseline settings and a manually set threshold at 0.1. Standard curve was constructed through serial dilutions of plasmids for quantification and checked for qPCR efficiencies.

2.4. Droplet digital PCR

The QX200™ Droplet Digital PCR System (Bio-Rad, Hercules, CA, USA) was used in the study. The total ddPCR reaction volume was 20 μL, containing 10 μL 2× ddPCR™ supermix for probe (no dUTP) (Bio-Rad, Pleasanton, CA, USA), 1 μmol L^{-1} of each primer, 500 nmol L^{-1} of probe, and 2 μL template DNA. Approximately 20 000 droplets were generated using a Droplet Generator (DG) with an 8-channel DG8 cartridge and cartridge holder with 70 μL of DG oil per well and 20 μL of reaction mixture. Following this step, 40 μL droplets mixtures were transferred into a 96-well plate. The PCR plate was heat-sealed using a PX1™ PCR Plate Sealer (Bio-Rad) and placed in the C1000 Thermal Cycler (Bio-Rad) under the following thermal conditions (temperature ramp rate 2°C s^{-1}): 95°C for 10 min, followed by 40 cycles of 94°C for 30 s and 54°C for 1 min. Droplets were counted on the QX200 droplet reader (Bio-Rad).

2.5. Data analysis

Linear regression analyses of standard curve from qPCR was performed and recalculated with Microsoft Excel Software (Microsoft, USA). Slope value of standard curve was used to determine PCR efficiencies. For ddPCR, positive droplets were discriminated from negative droplets by applying a fluorescence amplitude threshold with the QuantaSoft™ version 1.7.4 (Bio-Rad). Correlation analysis between ddPCR and qPCR was performed with SPSS Software version 21.0 (SPSS Inc., Chicago, USA). Pearson's correlations and linear regression were also used to evaluate the relationship between measurements of ddPCR and qPCR assays.

3. Results and discussion

Adequate discrimination between positive and negative signals is of great importance to set appropriate thresholds. Annealing temperature conditions play important roles in determining fluorescence intensity and the distance between positive and negative signals. To assess the optimal annealing temperature of the ddPCR assay, the eight