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BACTERIAL AND PHYTOPLASMA DISEASES

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Quantification of DNA of citrus huanglongbing pathogen in diseased leaves using competitive PCR

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Abstract A quantification system for huanglongbing pathogen using a competitive polymerase chain reaction method and image-analyzing software were developed to obtain precise results. Significant differences in the quantity of pathogen were thus determined in leaves of two citrus cultivars commonly cultivated in southern Vietnam. Less pathogen-related DNA was detected from the tissue of citrus cultivars that are believed to be more tolerant than susceptible cultivars. The quantification system will be used in studies on pathogen proliferation and movement inside citrus tissue.

Key words Huanglongbing · Citrus greening · *Candidatus* Liberibacter asiaticus · Competitive PCR · Quantification

Introduction

Huanglongbing (HLB, also known as citrus greening disease), one of the most severe diseases affecting citrus production in tropical and subtropical regions of the world, spread to citrus orchards in southern areas of Japan in 1988 (Miyakawa and Tsuno 1989). The distribution of this disease has now expanded in Japan from Okinawa Prefecture to Kagoshima Prefecture (Toguchi and Kawano 1997; Hamashima et al. 2003). The disease is usually diagnosed through symptom observation and detection by polymerase chain reaction (PCR) (Jagoueix et al. 1994), but these methods do not allow quantification of the pathogen. Quantification of the pathogen through methods such as dot-hybridization (Hung et al. 2000) and competitive PCR (Tian et al. 1996) has not been sufficiently precise to evaluate disease tolerance among citrus cultivars.

We have constructed competitor DNA and image-analyzing software for quantification of the HLB pathogen using competitive PCR (Wang et al. 1989) to quantify differences in the amount of the HLB pathogen among citrus cultivars.

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Materials and methods

Production of DNA standard

To verify the quantification system, amplified DNA fragment from the HLB pathogen with OI1/OI2c primers (Jagoueix et al. 1994) was cloned into a plasmid vector (pCR4-TOPO; Invitrogen, Carlsbad, CA, USA) using a TOPO TA Cloning Kit (Invitrogen). The vector DNA was used to transform *Escherichia coli* (TOP 10F') and amplified by culturing on LB medium (10 g bacto-tryptone, 5 g bacto-yeast-extract, 10 g NaCl per liter). Plasmid DNA extracted from *E. coli* was treated with RNase A and purified with a QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA). The concentration of DNA was determined by measuring the absorbance at 260 nm for use as a DNA standard.

Construction of competitor DNA

Competitor DNA for competitive PCR was made from a DNA fragment amplified by rep-PCR with DNA extracted from HLB-infected citrus (Bruijin et al. 1996; Kawabe and Onuki 2003). The PCR primer sequence for primer pair OI1/OI2c, designed for specific amplification of the 16S rRNA gene sequence from the HLB pathogen (Jagoueix et al. 1994), was attached to enable competitor DNA to be amplified competitively HLB-pathogen DNA. This constructed DNA fragment was cloned in the same way as the DNA standard. The size of the competitor DNA was 1000 bp, and the GC ratio was 51.3% (Fig. 1), whereas the

Fig. 1. Sequence of competitor DNA. *a*, Polymerase chain reaction (PCR) forward primer (OI1) binding region; *b*, PCR reverse primer (OI2c) binding region

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                                          a
  1 GCGCGTATGC AATACGAGCG GCAGGCGACG CTGACGGTAA CATGCATCGT
 51 AGAAGCCTTG TCGTCAGACT CGATGAACGG AATTCATAGG ATGAGCACTT
101 ACAAAATGTA CCAAGATCCT TGCTCTTGTA TTGCACACTC TGGTAGAGCA
151 GAGACTGTGG GATTTCATTG ATAGGCCTGC TTCCTATACT TGTACACACT
201 TTGACCTTTT GTTTGCTTGC CGACTATCGT CAATCTCAGG TACACCAGGA
251 TATGGCGCCT GCAGCTAGAG ACGACTTGCT TGACTTCGTA GGCTATAGTT
301 CGTGGTCCAT CTGCCACAGA AGAACCTGTC AAGATTGGTG AGGGTTGGCT
351 TCTGCTGGCC ATCTCGTGTG AAAGACGTAT GTGGGGAACA TTCACTCTTT
401 GCAACAACAA GAACCGGTAG GATGAAGAAG CTACCTGTGC CTCCGGAGTC
451 TTGACATTGT ATTTCGAAGC TCAGCGCCAT CCGCTGATTT GACTTGTAGG
501 AAGAAATATT GGACTCTGAT GTCGTAGTGC GAAGTGAAGT AGGGTTTGCG
551 GACGGGAGCG GATGTAAGAG TATTCGTGAG TGGAATGTAC TGCGCGTCGC
601 GACGTCGCGT TCCTGACGCG TACCGGAGGT CGATGCGACC AAGTTTGATT
651 GGTTGGACCA ACTGAGCTTA ACCCCAAGGT TCCCAGCCTC CCCTTGAATC
701 CAATGACCAG ACTCTGTTCG CATCACCGAC CGCCAGCTTG TGACAGCATG
751 CATCGTCACT ACATGAAACT CGGGGACTCA AACACATCTG TGATTACAGC
801 CTAGATTATC CTGGTAAAGC CCCACCACCT CTCGCCATTT TTCTCGCCCT
851 CTGATCACGA CGATTATGAT TCGCTCTTGA ACCCTTGCTC GCTTCCTTTC
901 TGTTCCTCGC TCTTTGAGTG CCAGTGTCAA ACGAAGGCCC TGCTACACTC
951 GCTCCGCCTC TACTTGGACC TCCACCGCAT GGGTTCGCAA GTCGCGAGGC
                                                      b
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size of the target-DNA sequence (accession number L22532, Jagoueix et al. 1994) was 1166 bp and the GC ratio was 52.8%.

Competitive PCR conditions

PCR amplifications were conducted with primer pair OI1/OI2c. The PCR reaction mixture (10 µl) contained 1× PCR buffer (Ex Taq buffer, Takara Bio, Otsu, Japan), 250 µM dNTPs, 200 nM of each primer, and 0.25 U *Taq* polymerase (TaKaRa Ex Taq, Takara Bio). A 0.5-µl sample of both the competitor DNA and target DNA (or DNA standard for system verification) were mixed into the PCR reaction mixture. Thermal conditions consisted of denaturation at 95°C for 2 min; 45 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min; final extension was at 72°C for 7 min.

Quantification of DNA

The amplified DNA was measured after competitive PCR using an ultraviolet (UV)-fluorescence image of the electrophoretic gel stained with ethidium bromide. The image was captured with a CCD camera (Printgraph AE-6911FX, Atto, Tokyo, Japan), and stored in bitmap format for MS-Windows. The image data was analyzed by computer software that we developed for this purpose, using a compiler (Delphi 7J professional; Borland Japan, Tokyo, Japan). With this software, the amount of amplified DNA is calculated from recorded signals of the pixel in bitmap, from top to bottom according to the movement of DNA molecules during electrophoresis. To subtract the background signal, the baseline was calculated, and the range of pixels conferring the DNA signal was determined. This range of signals was subtracted from the baseline and the cumulative signal was added to obtain the total amount of signal from DNA in the electrophoretic gel.

Collection of citrus tissue

To assess our quantification method, HLB-infected citrus leaves from two cultivars, Cam sanh and Nam roi, were collected from southern Vietnam. Twigs of citrus with more than five leaves were collected and the leaves used separately in the quantification procedure. Collected leaves were kept cool with a dewar vessel and processed for transportation from Vietnam to Japan according to the regulations of the Naha Plant Protection Station (Import Permit no. 15-NASHOKU-350). Besides young leaves that have the softness of the juvenile growth stage, five leaves were taken from the top of a twig. A length of midrib was cut out for pathogen quantification by first separating the leaf blade and petiole wing with a sharp razor blade. Second, the midrib was excised from the leaf blade, then the midrib was cut 1 cm from the bottom end of the midrib. The fresh mass of each excised midrib was then recorded. Each midrib was then divided into two pieces and put into 1 ml of 2× CTAB buffer [2% hexadecyltrimethylammonium bromide, 1.4 M NaCl, 100 mM Tris-HCl pH 8.0, 10 mM ethylenediaminetetraacetic acid (EDTA)] in a 2-ml plastic tube with a screw cap. Each closed tube was sealed with parafilm to prevent leakage during transport to Japan.

DNA extraction from citrus tissue

DNA was extracted from HLB-infected tissue by a cetyltrimethylammonium bromide (CTAB) method (Nakashima et al. 1998). The collected citrus tissue in the buffer in the screw-cap tube was transferred to a mortar. After grinding with a mortar and pestle, the juice was poured back into the same tube. The mortar and pestle was washed with 0.5 ml of the buffer, which was then added to the tube, then washed again with 0.5 ml of chloroform–isoamyl alcohol (24:1) and transferred to the tube. The

extraction then followed the conventional CTAB method, and after ethanol sedimentation, the DNA pellet was suspended in 50 µl of Tris-EDTA buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA). Because DNA was extracted from a 1-cm-long midrib and half was suspended in 50 µl, this solution was taken as the concentration of 1 cm (of midrib)/100 µl for quantification.

Application of DNA from citrus in competitive PCR

DNA extracted from citrus (1 µl) and 1 µl of 1 pM competitor DNA were mixed into each PCR tube to quantify pathogen DNA in the citrus tissue. Therefore, equal amplifications of two DNAs, competitor DNA and original citrus-extracted DNA, were calculated as 10 fmol/m (midrib length) of the pathogen.

Results and discussion

DNA quantification by competitive PCR is based on competitive amplification of two template DNAs, target DNA and competitor DNA, during execution of the PCR program. For this competitive amplification, we raised the number of cycles in our PCR program, originally designed to detect the HLB pathogen, to 45 for higher sensitivity and confirmed that this program was adequate for competitive PCR. The use of fewer cycles, however, is also applicable for competitive PCR according to the reaction scheme.

After PCR amplification, competitive PCR requires precise evaluation of competition between amplifications of target DNA and competitor DNA. The amplified DNAs are evaluated by image analysis of the products in an electrophoretic gel, so the imaging system is quite important for the precision of this quantification. Between the optical receiver and the data recorder, it is better to have minimal image-controlling apparatus, which controls gain, offset, and exposure time. The typical image-capturing apparatus for a commercial digital camera is not recommended for this reason, because most of them process images by modifying the signal strength of each pixel.

Increasing the exposure and contrast of the image of the electrophoretic gel after PCR gives clear results for detection purposes. However, an increase in exposure may cause signal saturation, thus losing the peak of the highest signal for the DNA. Therefore, moderate exposure is suitable for quantification. Our imaging software has been equipped with a function to check signal saturation to improve the reliability of quantification. An example of an electrophoretic gel image was captured (Fig. 2) with a 4-s exposure, partially overexposing the DNA image; a DNA band of 500 bp in the two rightmost columns shows overexposure. Signal intensities for the 500-bp DNA bands from this gel image and from two other images with shorter exposures (1 and 2 s) are graphically shown in Fig. 3, indicating complete overexposure of two DNA bands (500 bp) with a 4-s exposure time. Data for shorter exposures show an increasing linearity for the relationships between DNA amounts and

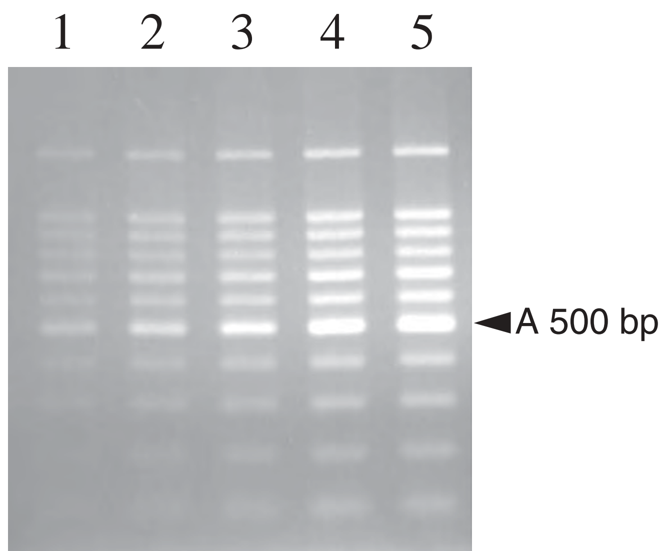

Fig. 2. Electrophoretic image for test of the relationship between DNA quantity and signal detection. *A*, DNA bands for correlation test. DNA size markers (100-bp DNA Ladder, Takara): lane *1*, 100 ng; lane *2*, 200 ng; lane *3*, 300 ng; lane *4*, 400 ng; lane *5*, 600 ng. Camera settings: focal length 51 mm, aperture f2.4, and exposure time 2.0 s

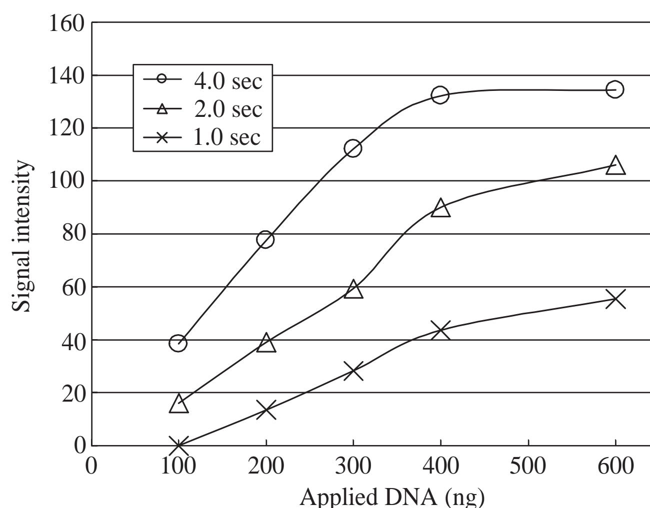

Fig. 3. Relationship between DNA quantity and detected signal for different exposure times. DNA used: 100-bp DNA Ladder (Takara)

signal intensities, but not complete linearity for higher amounts of DNA. This incomplete linear relationship is another problem that will affect quantification from the electrophoresis gel image. Because DNA bands with less DNA have almost complete linearity, overloading with DNA seems to cause this problem. Therefore, by eliminating both overexposure of image capturing and overloading with DNA, we can obtain signal intensities and DNA amounts that have a good linear relationship, enabling precise quantification of DNA with our imaging system. We also need to test other combinations of equipment, such as UV illuminators and CCD cameras, using verified amounts

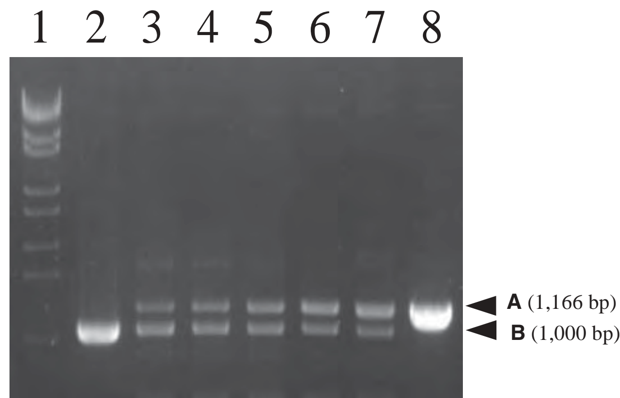

Fig. 4. Example of competitive PCR. *A*, amplified target DNA; *B*, amplified competitor DNA; lane *1*, DNA size markers (λ *Eco* T141); lane *2*, multiplication of competitor DNA; lanes *3–7*, competitive multiplication of target DNA and competitor DNA. Concentration of template DNAs are as follows: 1 pM target DNA and concentration of competitor DNA, 100 pM, 10 pM, 1 pM, 100 fM, and 10 fM, respectively. Lane *8*, multiplication of target DNA

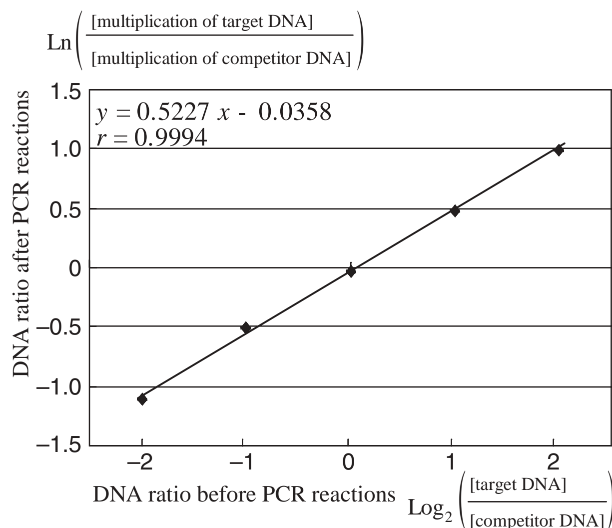

Fig. 5. Correlation between amounts of template DNA and PCR-amplified DNA

of DNA to evaluate the reliability of the DNA quantification.

For verification of our quantification system, a cloned DNA standard was used as the target DNA for the competitive PCR. With different amounts of the standard DNA, we obtained a competitive reaction based on the ratio of standard DNA to competitor DNA (Fig. 4). A logarithmic graph shows the linear relationship between ratios of the two DNAs before and after competitive PCR (Fig. 5). Based on this relationship, the quantity of HLB pathogen DNA can be calculated. Because amplification of the target DNA and competitor DNA occurs in the same tube, the competitive PCR should be less affected by any PCR-inhibitory substances in the reaction solution, whereas quantification by real-time PCR will be seriously affected by an inhibitory substance such as polysaccharides, which

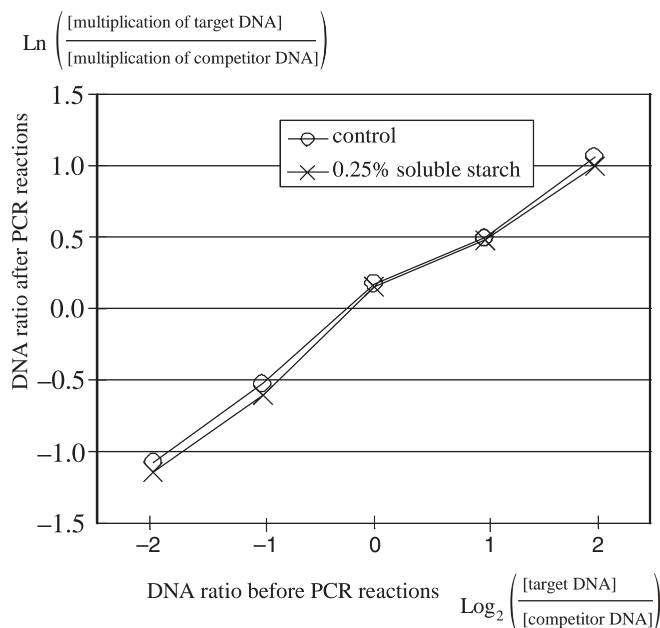

Fig. 6. Effect of soluble starch on competitive reactions between target DNA and competitor DNA

will appear in DNA extracted from plant tissue with the CTAB method. Because starch accumulates in HLB-infected leaves (Schneider 1968), starch is likely to contaminate DNA extracted from HLB-infected leaves, thus inhibiting the PCR. Only 0.5% soluble starch in a PCR reaction solution can completely inhibit DNA amplification under our PCR conditions, and 0.25% soluble starch mixed in a PCR reaction solution significantly decreases DNA amplification but will not seriously affect DNA quantification by competitive PCR (Fig. 6). The concentration of PCR inhibitory substance inside plant tissues will vary according to the plant part, growing conditions, and seasons, so competitive PCR will work for the comparison of DNA quantities from plant tissues under different conditions.

As an example of the application of our quantification system, infected citrus samples of two citrus cultivars, Cam sanh and Nam roi, were collected in southern Vietnam, where they are commonly cultivated. Cam Sanh belongs to the mandarin group, which is rather vulnerable to HLB disease, and Nam roi belongs to the pummelo group, which is rather tolerant (Garnier and Bové 2000; Chomchalow 2004). The quantities of the pathogen inside infected leaves can vary due to many variables such as soil, sunlight, fertilizer, etc. To minimize these effects and to clarify the difference between citrus cultivars, the samples were collected under predefined rules, such as taking just one twig from one orchard. A citrus tree was randomly selected for having typical symptoms of HLB disease, typical growth conditions in the orchard, and no physical damage from wounds or insects. From the selected tree, one twig was selected for having typical symptoms, receiving enough sunlight, growing upward, and not having any physical damage. The pathogen was quantified in collected samples using the competitive PCR method (Fig. 7). The results were calculated

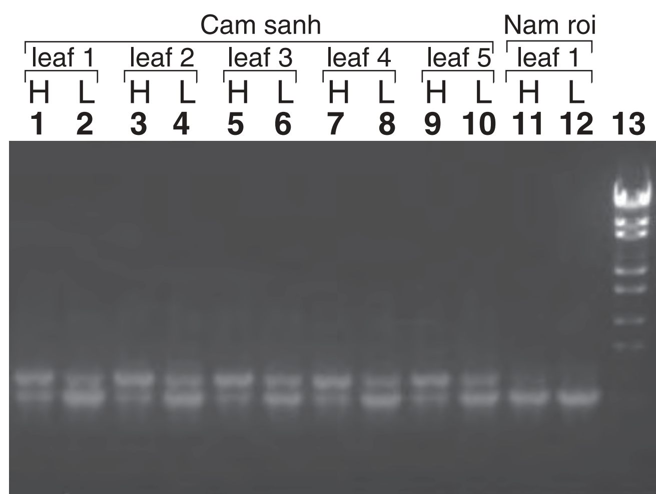

Fig. 7. Quantification of DNA of huanglongbing pathogen by competitive PCR from leaves of two citrus cultivars, Cam sanh and Nam roi, grown in southern Vietnam. *H*, 1 μl of DNA solution extracted from citrus midrib was mixed in each PCR tube; *L*, 1 μl of 13-times-diluted DNA solution extracted from citrus midrib was mixed in each PCR tube. Lanes *1–10*, competitive PCR of citrus cultivar Cam sanh; lanes *11* and *12*, competitive PCR of citrus cultivar Nam roi. Lanes *1–12*, 1 μl of 1 pM competitor DNA was mixed in each PCR tube; lane *13*, DNA size markers (λ *Eco* T141)

Table 1. Amount of huanglongbing pathogen in citrus cultivars

Cultivar	No. of leaves	Pathogen	
		fmol/m[a]	fmol/g Fresh mass[b]
Cam sanh	47	39 ± 26	35 ± 24
Nam roi	33	13 ± 10	6 ± 4
$P\ (T \leq t)$[c]		<0.0001	<0.0001

[a] Molar concentration per length of leaf midrib
[b] Molar concentration per fresh mass of leaf midrib
[c] *t*-Test between average pathogen molar concentrations in two citrus cultivars

for both length and fresh mass of the excised midrib. The average pathogen quantities in these citrus cultivars differed significantly by Student's *t*-test (Table 1). A comparison of two cultivars is not enough to say which calculation method, by fresh mass or by length of midrib, is better for evaluating disease tolerance. Further study is required to clarify the relationship between the lower amounts of the pathogen in the midrib of Nam roi and the longevity of this cultivar after HLB infection, such as analysis of other cultivars and quantifying the pathogen over time after artificial inoculation.

The quantification system that we developed will be used for further HLB disease analyses. This system has two advantages over the real-time PCR method that has also been used to quantify DNA. First, with amplification of both target DNA and competitor DNA occurring in the same tube, there is lower risk of error resulting from differences in amplification efficiencies caused by inhibitors, such as phenolic compounds and polysaccharides. Second, the equipment and chemicals required for our method are cheaper and more readily available than those used in real-time PCR. Real-time PCR requires a rather expensive machine and a reliable electricity supply, which may be difficult to obtain in some locations in the nations where many citrus-orchards suffer from HLB infection. In these nations, the fluorescent-dye-labeled PCR primers that are used for the real-time PCR method using the TaqMan probe may also be difficult to obtain. Our quantification system should work effectively for various uses, such as evaluating citrus HLB-disease tolerant cultivars, studying pathogen proliferation in both citrus plant and insect vector and their effect on insect transmissibility, and comparing pathogen quantities in different plant tissues that may contain varying levels of PCR inhibitory substances.

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