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MOLECULAR DETECTION OF THE POSSIBLE CAUSATIVE AGENT OF CITRUS GREENING DISEASE BY SINGLE PRIMER DNA AMPLIFICATION FINGERPRINTING

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ABSTRACT

A DNA sequence for a putative causative organism of citrus greening disease was detected by DNA Amplification Fingerprinting (DAF). Forty arbitrary oligonucleotide primers ranging from five to twelve nucleotides in length were screened for polymorphisms of DNA from greening disease infected and apparently healthy *Citrus reticulata* Blanco and *C. sinensis* Osbeck trees. One primer (5' GGGTAACGCC 3') generated a pronounced diagnostic band of 200 bp from infected plants which was not present in healthy trees. The diagnostic polymorphic marker band was excised from the silver stained polyacrylamide gel, cloned, and DNA sequenced. The sequence of the DNA amplification product from infected *C. sinensis* shows homology with bacteria. 200 base pairs are a small fragment of DNA to conclude a possible cause causative organism of Citrus Greening Disease (CGD) yet it shows maximum homology (>80%) with bacteria.

Additional Key Words: *Detection, Citrus-greening, Citrus reticulata, Citrus sinensis*

INTRODUCTION

Citrus greening disease (CGD) is a devastating disease problem causing extensive damage to citrus trees in many Asian and African countries. Infection by the greening pathogen becomes apparent only at the fruiting stage when it is beyond control. Fruits remain small, lopsided in shape and green on the shaded side, even after normal period of maturation. A phloem-colonizing microbe has been identified as a bacterium by electron microscopy (Garnier *et al.*, 1984). Recently, the bacterium was identified as *Liberobacter asiaticum* in Asian countries and *L. africum* in South Africa by cloning 16S rRNA gene (Jagoueix *et al.*, 1997). However, it has not yet been culturable in synthetic cell-free media. Accordingly, development of disease symptoms has not been demonstrated in citrus plants by any culturable causative orgnisms. The greening pathogen is transmitted in Asia by the insect *Diaphorina citri* Kuwayama (Regmi and Lama, 1988) as well as by means of planting and grafting materials (Jagoueix *et al.*, 1994).

Early diagnosis is not only a critical step towards applying efficient eradication and vector control measures when epidemics occur, but equally important as a measure to preserve germplasm collections of citrus. Over the past 20 years different techniques, such as serological and histochemical analysis, antibiotic sensitivity test, indexing techniques and electron microscopy have been used to detect possible causative agents of the greening disease (Da-Graca, 1991). These techniques are cumbersome and technically complex. Therefore, a rapid, low cost method for early detecting citrus greening disease without the use of labeled probes and obtaining pure culture is of utmost importance, especially if it is to be applicable to lesser developed countries.

Villechanoux *et al.* (1992) detected greening disease from different parts of Asia by DNA hybridization with three radiolabeled probes developed by differential hybridization between healthy

and diseased periwinkle plants, infected with the Poona strain bacterium-like organism. However, these clones did not hybridize with a South African strain. Sequence homology between one insert clone of greening pathogen and the *nus*G-*rpl*KAJL-*rpo*BC gene of bacteria (Villechoux *et al.*, 1993) was observed. However the technique developed by Villechanoux *et al.* (1992) required a high amount of pure DNA of the putative greening organism as well as labeled probes. Both requirements are difficult to achieve in developing countries where the problem is most pronounced.

In the present study we applied the DNA Amplification Fingerprinting (DAF) technique (Caetano-Anollés *et al.*, 1991) to detect amplification differences between samples with apparently healthy and symptoms of citrus greening collected from field. In recent years the DAF procedure has been successfully used as a diagnostic technique for distinguishing closely related genotypes of both eukaryotic and prokaryotic organisms (Caetano-Anollés *et al.*, 1991, 1997, Bassam *et al.*, 1992). A significant aspect of using short arbitrary primer for generation of fingerprints is that depending upon match of primer sequences, some primers amplify DNA from mixed DNA population. Eskew *et al.* (1993) differentiated *Azolla* and *Anabaena* which coexist in a symbiotic relationship using the DAF procedure. Gresshoff *et al.* (1998) demonstrated that DAF can distingusih mycorrhizal fungus from the host pine root. From this work it is evident that DAF can be used to generate separately fingerprints of plants and microorganisms living together under symbiotic or pathogenic conditions.

MATERIALS AND METHODS

Source of plant materials

Citrus leaves were collected from 10 to 12 year old trees from two different species. Greening disease was identified mainly by leaf and fruit symptoms.

Citrus reticulata Blanco (Mandarin):
I, no apparent infection observed, possible disease free plant (control)
II, greening infection apparent

Citrus sinensis Osbeck (Orange):
I, no apparent infection observed, possible disease free plant (control)
II, greening infection apparent

Six samples each of infected citrus species and two samples each of healthy plants were collected from the citrus orchard at the Horticulture Research Station Pokhara (900 m. asl and 200 Km west from Kathmandu), Nepal. Sample leaves of *C. reticulata* were also collected from a 10 - 12 year old tree and a 1 year old nursery plant from Lumle Agricultural Research Center (1,650 m asl) where the greening disease has not been observed so far. These samples were collected as control. All samples were fixed in 75% ethanol and transported to Knoxville TN, USA. Additional samples were obtained from Faisalabad, Pakistan and Stanleys Nursery (Knoxville). DNA from citrus leaf-mid ribs was isolated according to Dellaporta *et al.* (1983).

DNA amplification

The amplification reaction was performed in a total volume of 25 *u*l reaction mixture comprising of 3 *u*M heptamer, octamer and decamer or 30 *u*M pentamer (Integrated DNA Technologies, INC. Coralville, IA), 0.2 unit/□l of a truncated *Thermus aquaticus* AmpliTaq Stoffel fragment DNA Polymerase (Perkin-Elmer, Norwalk, Conn), 200 *u*M of each dNTP (United States Biochemical, Cleveland, OH), 10 mM Tris HCl pH 8.3, 10 mM KCl, 1.5 mM $MgCl_2$ and 1 to 2 ng of template DNA (Caetano-Anollés *et al.*, 1992). The amount of DNA before and after amplification was measured using a Fluorometer (TKO-100 Hoefer Scientific Instruments, San Francisco, CA). The amplification of reaction mixture overlaid with a drop of heavy mineral oil (Mallinckrodt, USP)

was carried out in a twin-block Thermocycler (Ericomp Inc., San Diego, CA). Thermocycling was carried with a temperature profile of two step cycles of 1 s at 96°C and 1 s at 30°C 35 cycles and 72°C for 5 minutes. Ericomp cyclers are of moderate ramping speed (13 - 18 °C per minute).

DNA separation and silver staining

DNA Amplification products were analyzed by polyacrylamide gel electrophoresis (PAGE) (Mini Protein Gel II, Bio Rad). Polyacrylamide gel electrophoresis was carried out in 0.45 mm thick gel slab of 4.5% polyacrylamide with a 19:1 ratio of acrylamide to the cross-linker piperazine diacrylamide (Bio Rad), 7 M Urea, TBE (100 mM Tris HCl, 83 mM boric acid and 1 mM Na_2EDTA pH 8.3), 10% ammonium persulfate and N,N,N'.N'-Tetra-methylethylene- diamine (TEMED). Gels were usually cast onto a gel bond PAG polyester backing film (FMC, Rockland, ME) which was used to support the gel and for further handling. Sample (3.5 *ul* of approximately 30 ng/*ul*) was loaded with equal amount of loading buffer (5 M urea and 0.02% xylene cyanole FF) in gels which has been prerun for about 10 min in 1 M TBE electrophoresis buffer (Caetano-Anollés *et al.*, 1991). Electrophoresis was carried out at 118 V for approximately 75 minutes.

After electrophoresis the gels were treated with 7.5% acetic acid for 15 min, rinsed in double distilled water three times for two minutes, impregnated in $AgNO_3$ (1 g/liter), 1.5 ml 37% HCOH/liter for 25 min rinsed quickly with water and developed by Na_2CO_3 (30 g/liter), 2.5 ml 37% HCOH/liter, $Na_2S_2O_3.5H_2O$ 2 mg/l) at 8-10°C. The staining reaction was stopped by using cold 7.5% acetic acid (Bassam *et al.*, 1991, Caetano-Anollés and Gresshoff, 1994) and gels were air-dried for preservation.

Isolation, cloning and sequencing of the disease specific DNA amplification product

A 200 bp band size was excised from PAGE gels and subsequently reamplified with the same primer (5 GGGTAACGCC 3) in a 25 *ul* reaction volume (Weaver *et al.*, 1994), six times, to produce a single copy band. The amplified DNA product (100 ng) in 7.5 *ul* water was treated with 1 *ul* of 10x ligation buffer, 1 *ul* of 10x dNTP (1 mM each), and 0.5 *ul* Klenow fragment, incubated at 37°C for 30 minutes for filling blunt ends of DNA for cloning. Klenow fragment was deactivated at 65°C for 10 minutes. Recently, we developed a more efficient band reisolation method (Men and Gresshoff, 1998).

The DNA fragment was cloned into the *Sma*I site of vector pGEM-3Z according to Promega Technical Bulletin, transferred into JM109 strain bacteria (Promega) and white colonies containing recombinant plasmids were selected from Isopropylthio- D-galactoside (IPTG) and 5-Bromo-4-chloro-3-indolyl -D-galactosidase (Xgal) containing media. Plasmid DNA of the pGEM-3Z vector was digested with *Xba*I and *Kpn*I, blotted onto Zeta probe blotting paper (Bio Rad) and hybridized overnight at 65°C with a ^{32}P- *a*dATP labelled amplification product probe, using the random primer labelling kit (Boehringer, Germany)

Plasmid DNA was isolated from 10 ml overnight culture according to the mini-preparation protocol, extracted once with Phenol/ Chloroform/ Isoamyl and once with Chloroform/Isoamyl Alcohol (Sambrook *et al.*, 1989). DNA was precipitated by 0.1 volume of 3 M NaOAc (pH 4.8) and 0.6 volume of cold isoproponol (-20°C) and incubated in liquid nitrogen for 10 minutes. DNA was pelleted at 4°C and dissolved in 1 ml water, precipitated with 0.5 ml of 30% PEG-8000 in 30 mM $MgCl_2$, incubated 10 minutes at room temperature and spun down at maximum speed for 10 minutes (Nicoletti and Condorell, 1993). The PEG-precipitated DNA was denatured by 2 M NaOH, 2 mM EDTA at room temperature for 5 minutes and neutralized with 3 M NaOAc pH 4.8 for sequencing (Wang and Sodja, 1991). The sequencing reaction was done using the M13 reverse primer, ^{32}P-*a*dATP (3000 ci/mmol DuPont NEN, Boston, MA) and sequenase version 2 according to protocol supplied by United States Biochemical (Cleveland, OH). The reaction product was separated in 5%

acrylamide and 7 M urea in a PhorRunner sequencing gel system (Jordan Scientific Co. Bloomington IN). The gel was treated twice with 10% acetic acid at intervals of 20 minutes, dried in air for 4 to 6 hour and exposed to Kodak X-ray film.

RESULTS

DNA amplification fingerprints of citrus species

Figure 1 shows polymorphic DAF patterns between different citrus species *Citrus reticulata* from Knoxville (TN), *C. reticulata* from Nepal, *C. limon* from Pakistan, *C. sinensis* from Nepal and Pakistan amplified with arbitrary oligonucleotide primer (5' CCTGGTCG 3'). DAF clearly differentiated between *C. reticulata* from Nepal and from the Knoxville nursery as well as *C. sinensis* from Nepal and Pakistan.

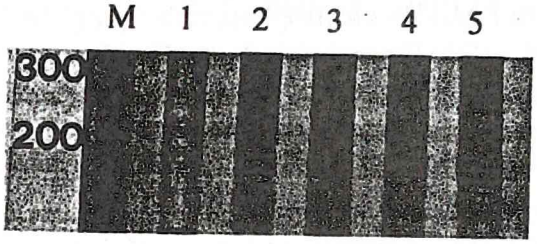

Figure 1. Genomic DNA profiles of citrus species from Nepal and Pakistan as amplified by arbitrary primer (5'CCTGGTCG 3'): Lane 1, *Citrus reticulata* (Nepal); Lane 2, *C. reticulata* (Knoxville Nursery); Lane 3, *C. lemone* (Pakistan); Lane 4, *C. sinensis* (Nepal); Lane 5, *C. sinensis* (Pakistan). M (Marker (bp).

DNA amplification fingerprints of infected and non-infected plants

Forty arbitrary oligonucleotide primers ranging in length from five to twelve nucleotides were screened for finding diagnostic polymorphism in infected and apparently healthy orange and sweet orange plants from Nepal. Among 40 primers, half yielded polymorphic banding patterns between *Citrus sinensis* and *C. reticulata* (data not shown). We observed monomorphic banding patterns between infected and non-infected plants with 39 primers (data not shown). The average amplification profile showed 25 silver stained bands of different staining intensity. The normal range of observable products was from 100 to 1,000 bp.

One of the primers (5' GGGTAACGCC 3') generated a pronounced diagnostic band of 200 bp size in both infected plants of mandarin and orange which was not observed in non-infected plants of the two different species (Fig. 2a). However, there was no sharp difference between fingerprint patterns of infected and non-infected plants when separated in 1.2% agarose gel (data not shown). To confirm whether this diagnostic marker band was a specific product of the DNA polymerase Amplitaq Stoffel Fragment, which lacks the 5'-3' exonuclease activity (Guide to PCR enzymes: Perkin/Elmer), we further amplified DNA of infected and non-infected plants with AmpliTaq polymerase and with the same primer under identical condition. The same diagnostic marker band was confirmed in silver PAGE gels. However, AmpliTaq polymerase yielded an additional polymorphic band of 250 bp in size in infected plants (Fig. 2b). The origin of this band was not further investigated.

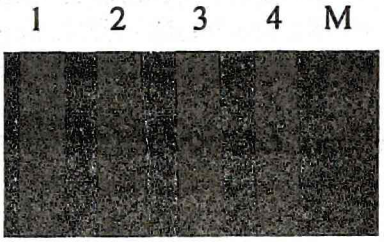

Figure 2 (a). DNA profiles of greening disease infected (I) and non-infected (NI) citrus plants: Lane 1, *C. reticulata* (NI); Lane 2, *C. reticulata* (I); Lane 3, C. *sinensis* (NI) and Lane 4, *C. sinensis* (I). Genomic DNA of these plants was amplified with Amplitaq Stoffel Fragment and 5'GGGTAACGCC 3' primer. The line in between lanes is drawn for quick detection of band of interest. M (Marker-200 bp)

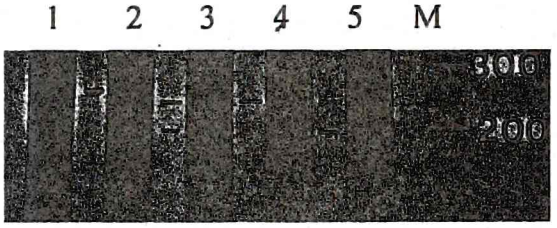

Figure 2 (b). DNA profile of greening disease infected and non-infected: Lane 1 *C. reticulata* (NI), Lane 2, *C. reticulata* (I), Lane 3, *C. sinensis* (NI), Lane 4, *C. sinensis* (I), 5 Lane, *C. reticulata* (I) Pakistan, amplified with Amplitaq DNA polymerase and primer (5'GGGTAACGCC 3'). The line in between lanes is drawn for quick detection of band of interest. M (Marker bp).

It was necessary to confirm whether the polymorphic band (200 bp) both in infected mandarin and orange (Fig. 2a) was a product of the pathogen and not of host plant. Therefore, the amplified DNA was sequenced as described by Weaver *et al.*, 1994. Sequence homology of the 200 bp fragment was searched by using gene bank BLASTn program. The maximum percentage (>80%) of similarity was found with bacteria.

DISCUSSION

There are no typical symptoms of citrus greening disease for field identification. Zinc and iron deficiency and other pathogens also duplicate greening symptoms. A potentially causative pathogen for citrus greening disease so far was only confirmed by electron microscopy (Villechanoux *et al.*, 1992) and by cloning (Jagoueix *et al.*, 1994 and 1997). Here we have demonstrated that a putative pathogen causing citrus greening disease could be identified by DNA amplification fingerprinting.

The use of silver stained polyacrylamide gel for analyzing DNA amplification products resulted in high resolution of especially lower sized fragments (50-300 bp) of both major and minor bands with a consistent reproduction in banding pattern. However, we could not detect such small amplification products in 1.2 % agarose gels. The sensitivity and reproducibility of the DAF technique for generating fingerprinting of bacteria, fungi and plant and bacteria in symbiotic

condition has been successfully demonstrated by several workers (Eskew *et al.*, 1993, Gresshoff and MacKenzie, 1994, Men and Gresshoff, 1998).

The presence of reproducible and consistent patterns of fingerprints between healthy and infected plants permitted the detection of disease-related DNA amplification products (200 bp) (Figure 2a). These were detected only in rare situations (i.e., one out of 40 primers). The disease-specific diagnostic band (200 bp) is not only observed in infected samples collected from Nepal but also found in citrus greening infected samples (*C. reticulata*, *C. sinensis* and *C. limon*) obtained from Pakistan (data not shown).

It was possible to clone the specific DAF band by repeated amplification. The clone (pCGD200) was sequenced to determine whether the DAF product contained the primer sequence (5'GGGTAACGCC 3') (Figure 3) and the degree of homology with other plant pathogens. The sequence of pCGD200 showed 80% homology with sequences of bacteria.

GGGTAACGCC ATTGGTGTGA AGCGTGATGC TGACGGGTGA CGATGACGGA
TGCGAGGTAT GCTTGCGAGT GAATAGACAT ATTGCTGTAA CCATAACATC
TGCACGTACG CTATGGACAT TTAGGGTATT TGACTTGAAC CCGCAATGGG
ACCCATGGCT CGAGCTTAAG CGGATATCAC TCAGTCATGT .

Figure 3. Sequence of pCGD 200 bp cloned fragment. Sequence of DNA amplification product with primer (5'GGGTAACGCC 3') from genomic DNA of Citrus Greening Disease infected *Citrus sinensis* cloned in pGEM-3z vector

An ecological association of the greening pathogen with *tristeza virus* is often observed in Asia (Villechanoux *et al.*, 1992) as well as in Africa (Da-Graca, 1991). Citrus trees under field condition usaully harbour mixed infections of viruses (Lafleche and Bove, 1970), bacteria (Garnier *et al.*, 1984) including citrus canker pathogen, viriods, and fungi but viruses in mixed infections are quite common and may show the synergistic effect. Bhagabati and Nariani (1980) reported that an RNA agent is always associated with greening diseased citrus trees in areas where aphid-transmitted *tristeza* virus is endemic. To detect the possible causative organism of citrus greening disease, DNA from healthy and diseased samples collected from field grown citrus plants was amplified with a single short primer. A polymorphic band (200 bp) was cloned, sequenced and searched for sequence homology in gene bank. The CGD200 clone showed maximum homology with the sequence of bacteria. Although a 200 bp sequence is not sufficient to draw a conclusion for a causative organism, genus and species of CGD, it is obvious that the sequence showed maximum similarity with bacteria. The causative organism of CGD, the bacterium was identified as *Liberobacter asiaticum* in Asian countries and *L. africum* in South Africa by cloning 16S rRNA gene by Jagoueix *et al.* (1997). Our results, sequence homology of 200 bp polymorphic band from the sample obtained from Pokhara also indicate that the causative organism is bacteria.

The cloning and sequences of a DNA product derived from a citrus greening disease associated pathogen provides the opportunity to generate a PCR test. Such primer set would be useful for germplasm screening in Asia and Africa. Furthermore, such diagnostic test would permit the screening of large population at a low cost and rapid detection of CGD for quarantine purpose.

DNA of the putative organism of CGD was also detected by amplifying with 16S rRNA with specific PCR primer (forward primer 5'-ACGAAAGCGTGGGGAGCAAA-3' and reverse primer 5'-GAAGTCGAGTTGCAGACTTC-3') to detect DNA of a potentially causative pathogen in infected material as described by Ahrens and Seemuller (1992). (data not shown). There was only one product in infected plants; the product (588 bp) showed no hybridization with total genomic DNA of

healthy samples from different regions which indicates that the product was from DNA of causative organism. Since an amplification product (588 bp) was observed only in infected plant, we hybridized the product with five non-infected samples from Pokhara, Lumle and Knoxville and two infected plants of *C. reticulata* and *C. sinensis*. Southern blot hybridization of the ^{32}P labeled 588 bp PCR product to total genomic DNA from infected and healthy citrus trees showed preferential hybridization only to infected samples and virtually no signals in healthy plants (data not shown). Our result clearly suggests one type of causative organism which causes severe damage to citrus plants in South East Asia .

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