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Molecular and Cellular Probes 19 (2005) 173–179

Molecular and Cellular Probes

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Citrus huanglongbing in São Paulo State, Brazil: PCR detection of the '*Candidatus*' Liberibacter species associated with the disease

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Received 16 September 2004; accepted for publication 12 November 2004

Abstract

Symptoms of huanglongbing (HLB), one of the most serious diseases of citrus in Asia and Africa, have been noticed in March 2004 in the Araraquara region of São Paulo State, Brazil. HLB has not been reported previously from America. The causal HLB bacteria, *Candidatus* Liberibacter africanus in Africa and *Candidatus* Liberibacter asiaticus in Asia, can be detected in symptomatic citrus leaves by PCR amplification of their 16S rDNA with previously described primers. When this technique was applied to 43 symptomatic leaf samples from the Araraquara region, all PCR reactions were negative. This suggested that a new pathogen, not detected by the above primers, could be involved in HLB in the State of São Paulo. Indeed, by using universal primers for amplification of bacterial 16S rDNA, a new liberibacter species, *Candidatus* Liberibacter americanus, has recently been identified. Specific primers for PCR amplification of the 16S rDNA of *Ca.* L. americanus have been selected. Using these primers, the new liberibacter could be detected in 214 symptomatic leaf samples tested. The leaves of two additional samples were infected with *Candidatus* Liberibacter asiaticus, and two further samples contained both *Ca*. L. americanus and *Ca*. L. asiaticus. The samples came from 47 farms in 35 municipalities. The psyllid vector of *Ca.* L. asiaticus, *Diaphorina citri*, is established in South, Central, and North America (Florida and Texas). *Ca.* L. americanus could be detected by PCR in several batches of *D. citri* psyllids collected on symptomatic sweet orange trees infected with *Ca.* L. americanus, strongly suggesting that *D. citri* is the vector of *Ca.* L. americanus. The results reported here confirm the presence of HLB in the State of São Paulo. *Ca.* L. americanus is the most widely distributed pathogen.

Keywords: Citrus; Huanglongbing; Greening; Liberibacter; 16S rDNA; Brazil

1. Introduction

Huanglongbing (HLB), previously called greening, is one of the most serious diseases of citrus. The causal agent is a non-cultured, sieve tube-restricted member of the α-subdivision of the *Proteobacteria*: *Candidatus* Liberibacter africanus in Africa and *Candidatus* Liberibacter asiaticus in Asia [1]. HLB has not been reported previously from America. However, the Asian psyllid vector of *Ca.* L. asiaticus, *Diaphorina citri*, is established in South, Central, and North America (Florida and Texas). The insect reached Brazil 60 years ago, but entered Texas only in 2001. In March 2004, leaf and fruit symptoms resembling those of HLB were observed in several sweet orange (*Citrus sinensis* (L.) Osbeck) orchards in the Araraquara area of São Paulo State. Leaf mottling or 'blotchy mottle' [2], a characteristic feature of HLB, was the major foliar symptom. Fruits were small and lopsided, and contained many aborted seeds [3].

A PCR method has been described previously, and permits the detection of the two liberibacters in citrus leaves by amplification of an 1160 bp fragment of their 16S rDNA [4]. With both liberibacter species, the size of the amplicon is

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doi:10.1016/j.mcp.2004.11.002

the same (1160 bp). However, the *Ca.* L asiaticus amplicon contains one *Xba*1 restriction site, and yields two fragments (640 and 520 bp) upon restriction, while *Ca*. L. africanus has two such sites, and yields three fragments (520, 506 and 130 bp). It is thus easy to identify the liberibacter species involved [4]. The PCR method has been assayed in many Asian and African countries for the detection of the two HLB liberibacters [see for instance 5–7]. Whenever leaves with the classic blotchy mottle symptoms were used, positive PCR reactions were always obtained, and yielded the characteristic 1160 bp amplicon. In April 2004, it was decided to use the PCR technique to confirm the presence of HLB in São Paulo State, and identify the liberibacter involved: *Ca*. L. asiaticus or *Ca*. L. africanus. Unexpectedly, none of the symptomatic leaf samples from 43 affected trees, of which many had severe fruit symptoms, yielded a positive PCR test, under conditions where symptomatic control leaves infected with *Ca*. L. asiaticus or *Ca*. L. africanus from the HLB collection in Bordeaux, gave positive PCR reactions. However, at the same time, and using the same PCR technique, *Ca*. L. asiaticus was detected in two of 22 leaf samples (M.A. Machado, Cordeiropolis, S.P., Brazil, personal communication). In view of the many negative PCR reactions witnessed, the presence of a new bacterial pathogen in the symptomatic, blotchy mottle leaves from São Paulo State was suspected and investigated. This work has resulted in the discovery of a third liberibacter species, for which we have proposed the name *Candidatus* Liberibacter americanus [3]. The complete characterization of the new liberibacter is in progress. In the work reported here, primers for PCR amplification of the 16S rDNA of *Ca*. L. americanus have been used to detect the new liberibacter in citrus leaves, and evaluate its distribution, in comparison with that of *Ca*. L. asiaticus and *Ca*. L. africanus, within the huanglongbing-affected region of São Paulo State. Detection of the new liberibacter in *D. citri* psyllids was also attempted.

2. Materials and methods

2.1. Plant material

Two-year-old 'Hamlin' sweet orange seedlings infected with *Ca*. L. africanus or *Ca*. L. asiaticus were from the HLB collection in Bordeaux, France, and served as positive control material. Healthy and infected seedlings were maintained in a greenhouse as previously described [4].

Symptomless citrus leaves and leaves with characteristic blotchy mottle symptoms were collected in April, June and August 2004, on trees from 47 citrus farms within the 35 municipalities of São Paulo State where symptoms of HLB had been detected. Each leaf sample came from a single tree. When symptomatic leaves were sampled on trees bearing fruit, fruit symptoms of HLB always accompanied the leaf symptoms. Leaves with symptoms other than HLB blotchy mottle were also sampled. Each leaf sample contained 10–20 leaves. Leaves were kept in plastic bags at 4 °C before they were used for DNA extraction within 48 h [8]. In total, 271 leaf samples were collected. Most symptomatic leaf samples came from sweet orange trees, the major citrus cultivar grown in São Paulo State, but some were also collected on affected Ponkan mandarin, Cravo mandarin, and Murcott tangor trees.

2.2. Capture of D. citri psyllids

D. citri psyllids were captured with a motor-driven aspirator on three fully infected, symptomatic 'Pera' sweet orange trees, after PCR assays had shown the trees to be infected with *Ca*. L. americanus only.

2.3. PCR detection of Ca. L. americanus

PCR primers specific for amplification of the 16S rDNA of the new liberibacter were selected from the 16S rDNA sequence (GenBank accession number: AY742824). Fig. 1 shows the sequence of forward primer (GB1) and reverse primer (GB3) in comparison with corresponding sequences of *Ca*. L. asiaticus and *Ca*. L. africanus 16S rDNAs. The use of these primers leads to a 1027 bp amplicon. The PCR reaction was performed in 40 μl of reaction mixture containing 1 μM of each of the primers, 200 μM of each of the four dNTP, 2 mM $MgCl_2$, 20 mM Tris–HCl pH 8.4, 50 mM KCl, 1.5 U of *Taq* polymerase (Promega), and 1 μl of DNA preparation. A mastercycle gradient thermocycler (Eppendorf) with the following program was used for DNA amplification: 35 cycles each at 94 °C for 45 s, 64 °C for 45 s, and 72 °C for 60 s. Following amplification, 10 μl aliquots of each reaction mixture were analysed by electrophoresis on 1.2% agarose gels.

2.4. PCR detection of Ca. L. africanus and Ca. L. asiaticus

PCR was performed according to [4] with 35 cycles each at 92 °C for 40 s, and 72 °C for 90 s (annealing and primer extension at same temperature). The reaction mixture was the same than that for *Ca*. L. americanus. As shown on Fig. 1, the sequence of reverse primer OI2c is the same for both liberibacters. The sequences of forward primer OA1 for *Ca*. L. africanus and OI1 for *Ca*. L. asiaticus are identical except that GCA in OI1 is replaced by TTT in OA1. Both forward primers were used in the reaction mixture to favor amplification of either one of the two liberibacters [4]. Following amplification, the reaction mixture was analysed on 1.2% agarose gels. The amplified DNA has a size of 1160 bp for both liberibacters. However, the 1160 bp amplicon from *Ca*. L. asiaticus has one *Xba*1 restriction site and yields two fragments upon digestion (640 and 520 bp), while *Ca*. L. africanus has two such sites, and gives three fragments (520, 506, and 130 bp) [4]. The *Xba*1 test was used to identify the liberibacter involved.