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Production and evaluation of non-radioactive probes for the detection of the two '*Candidatus* Liberobacter' species associated with citrus huanglongbing (greening)

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The production and evaluation of non-radioactive probes for the detection of '*Candidatus* Liberobacter asiaticum' and '*Candidatus* Liberobacter africanum', the two bacterial species associated with citrus huanglongbing (greening) disease is described. Two DNA fagments, In 2·6 and AS 1·7, obtained previously from the β operons of '*Candidatus* Liberobacter asiaticum' and '*Candidatus* Liberobacter africanum', respectively, were the starting materials for production of the two non-radioactive probes. These digoxigenin (DIG)-labelled probes were generated by PCR incorporation of DIG-11-dUTP, yielding In 1·7-DIG and AS 1·7-DIG. Probe In 1·7-DIG was hybridized with DNAs extracted from 24 field-collected samples in Bali (Indonesia). The membrane on which the DNAs were blotted was first hybridized with radioactive probe ^{32}P-In 2·6. After the hybridization results were recorded, the radioactive probe was removed, and the membrane hybridized with DIG-labelled probe In 1·7-DIG. Identical results were obtained for 23 samples. One sample was positive with the DIG-labelled probe and negative with the ^{32}P-labelled probe. However, cross-hybridization of In 1·7-DIG with DNA from L. africanum was higher than that obtained with the radioactive probe. This cross-hybridization could be eliminated by raising the temperature of the stringent washing step. No field samples from Africa being available, probe AS 1·7-DIG was dot-blot hybridized against DNAs extracted from leaves of greenhouse-kept citrus plants from different geographical origins and infected with one or other Liberobacter species. The data showed that AS 1·7-DIG hybridized with L. africanum with a sensitivity equivalent to that of the radioactive probe.

KEYWORDS: citrus, huanglongbing, greening, Liberobacter, hybridization, digoxigenin.

INTRODUCTION

Huanglongbing (HLB), previously called greening, is a very severe disease of citrus in Asia, south-east Asia, south and eastern Africa and the Arabic Peninsula.[1,2] Symptoms of the disease are characteristic but not specific, hence the need for good detection methods of the uncultured bacterial species '*Candidatus* Liberobacter asiaticum' and '*Candidatus* Liberobacter africanum' associated with the disease.[3] In 1992 we were able to clone a 2·6-kbp fragment of the genome of '*Candidatus* Liberobacter asiaticum',[4] which cor-

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responds to several genes of the β operon.[5] In 1995, using PCR, we amplified and cloned a 1·7-kbp fragment within the β operon of '*Candidatus* Liberobacter africanum'.[66] When radiolabelled with ^{32}P and used as probes at high stringency, the two fragments proved to be efficient for the detection by dot-blot hybridization of the respective Liberobacter species from which they were obtained. However, the use of radioactively-labelled probes is not easy in most of the countries where HLB is present, and this is why we investigated the efficiency of non-radioactive, digoxigenin-labelled probes to detect the two liberobacter species. The most convenient way to label the probes with digoxigenin is PCR incorporation of digoxigenin-11-dUTP (DIG-UTP), as this generates vector-free probes quickly and with high yield.[7] However, PCR incorporation of DIG-UTP in large size DNA is sometimes difficult and, indeed, we failed to label the In 2·6-kbp fragment. Thus, we amplified by PCR and cloned a 1·7-kbp fragment from In 2·6 of '*Candidatus* Liberobacter asiaticum'. In this paper we show that the two DIG-labelled fragments, In 1·7 and AS 1·7, from '*Candidatus* Liberobacter asiaticum' and '*Candidatus* Liberobacter africanum', respectively, can be used for the detection of the two Liberobacter species with results equivalent to those obtained with the corresponding radioactive probes In 2·6 and AS 1·7.

MATERIALS AND METHODS

Plant material

Periwinkle (*Catharantus roseus* L.) and sweet orange (*Citrus sinensis* osb.) plants infected with different geographical strains of '*Candidatus* Liberobacter asiaticum' and '*Candidatus* Liberobacter africanum' were maintained in our greenhouse in Bordeaux.

The culture conditions for healthy and infected plants and the origin of the liberobacter strains used were described previously.[4]

Twenty-four leaf samples were collected in various orchards during a survey in Bali, Indonesia, in 1995, carried to Bordeaux and used for DNA extraction.

Extraction of DNA from plants

For each sample, DNA was purified from 2 g of leaf midribs from healthy or infected plants by the CTAB (hexadecyl trimethyl ammonium bromide) method of Murray and Thompson.[8]

Production of liberobacter-specific digoxigenin-labelled DNA probes

Cloning

The two primers 1898 and 1897 previously described[6] were used for PCR amplification of a 1·7-kbp fragment from the recombinant M13mp18 vector containing DNA fragment In-2·6 from '*Candidatus* Liberobacter asiaticum'.[4]

The amplified fragment, named In 1·7, was purified with the Wizard PCR preps system (Promega) and ligated in pGEM-T vector (Promega) according to manufacturer's instructions. One microlitre of the ligation mixture was used to transform competent *E. coli* XL1-Blue cells by high voltage electroporation.[9]

The sequence of clone In 1·7 was determined with a T7 Sequencing Kit (Pharmacia) and compared to that of In 2·6[5] to confirm the nature of the fragment.

For homogeneity, fragment AS 1·7 from '*Candidatus* Liberobacter africanum', which had been cloned in pUC 18 plasmid,[6] was recloned in the pGEM-T.

PCR

Probe-labelling was carried out by incorporation of digoxigenin-11-dUTP (DIG-UTP) during PCR reaction as follows: 50 µl reaction mixture containing 0·5 µM of each primer (1898 and 1897), 200 µM each of dATP, dGTP, dCTP, dTTP, 20 µM of DIG-11-dUTP (Boehringer Mannheim), 78 mM Tris–HCl pH 8·8, 17 mM $(NH_4)_2SO_4$, 10 mM β-mercaptoethanol, 2 mM $MgCl_2$, 0·05% W1 detergent (Gibco BRL), 200 µg ml^{-1} BSA, 1 ng of template plasmid and 2·5 units of *Taq* polymerase (Gibco BRL) were covered with paraffin oil and subjected to 35 cycles each at 92°C for 45 s, 50°C for 45 s, and 72°C for 2·5 min in a thermocycler (Thermojet, Eurogentec).

Eight microlitres of the PCR mixture was analysed by electrophoresis on 1% agarose gels and the DNA stained with ethidium bromide. The band corresponding to the digoxigenin-labelled amplicon was cut out of the gel and purified with the Geneclean II kit (Bio 101, Inc.). Yield of digoxigenin-labelled probes was estimated as recommended by the manufacturer (Boehringer Mannheim).

Radioactive labelling of Liberobacter probes

Fragment In 2·6 from '*Candidatus* Liberobacter asiaticum' was labelled with ^{32}P α[dATP] using the Random Primers DNA Labelling System (Gibco, BRL) as

described previously.[4] Fragment AS 1·7 from '*Candidatus* Liberobacter africanum' was separated from pUC 18 by enzymatic digestion (*Bam*Hl/*Kpn*l), purified and labelled as described above.

Dot-blot preparation

Fifteen microlitres of plant DNA were denatured with 50 mM methylmercury for 10 min at room temperature. The mixture was blotted with a filtration manifold system (Gibco, BRL) onto a positively-charged nylon membrane (Schleicher and Schuell), previously equilibrated in 5X SSC (1X SSC: 0·15 M NaCl, 0·015 sodium citrate, pH 7). The membrane was put on Whatman 3 MM paper presoaked in 0·4 M NaOH, for 20 min and briefly washed in 2X SSC.

Hybridization procedure

Membranes with the dotted DNAs were prehybridized for 2 h at 42°C in hybridization buffer (5X SSC, 0·5% SDS, 0·1 M Tris–HCl pH 8, 5X Denhardt, 50% formamide). The probe was denatured for 10 min at 100°C, chilled on ice, and added to the hybridization solution at a concentration of 15 ng ml^{-1} in the case of DIG-labelled probes or 0·8X 10^6 cpm ml^{-1} for ^{32}P-labelled probes. Hybridization was carried out overnight at 42°C. The membranes were washed three times in 2X SSC–0·5% SDS for 10 min at room temperature and 45 min in 0·1X SSC–0·1% SDS at 55°C. In some experiments, the last stringent washing was done at 65°C.

After hybridization with the radioactive probes, the membranes were autoradiographed as described previously.[4]

Membranes hybridized with DIG-labelled probes were treated for chemiluminescent detection with CSPD [disodium 3-(4-methoxypiro (1,2-dioxetane-3, 2′-(5′-chloro) tricyclo [3.3.1.1^{3,7}] decan)-4-yl)phenyl phosphate] (Boehringer, Mannheim) following the manufacturer's instructions. Briefly, the membranes were washed in buffer 1 [0·1 M maleic acid, 0·15 M NaCl pH 7·5 with 0·3% (v/v) Tween 20], incubated for 30 min in buffer 2 [0·1 M maleic acid, 0·15 M NaCl pH 7·5 with blocking reagent (1:10)] and for 30 min in diluted DIG-alkaline phosphatase conjugate (1: 10 000 in buffer 2). After two washes in buffer 1 for 15 min, the filters were equilibrated for 2 min in buffer 3 (0·1 M Tris-HCl, 0·1 M NaCl, 50 mM $MgCl_2$ pH 9·5) and incubated in diluted CSPD substrate (1:100 in buffer 3) for 5 min. Membranes were left to drip, sealed in plastic bags and incubated for 5 to 15 min at 37°C before exposure on X-ray (Konica) for 15 to 90 min at room temperature.

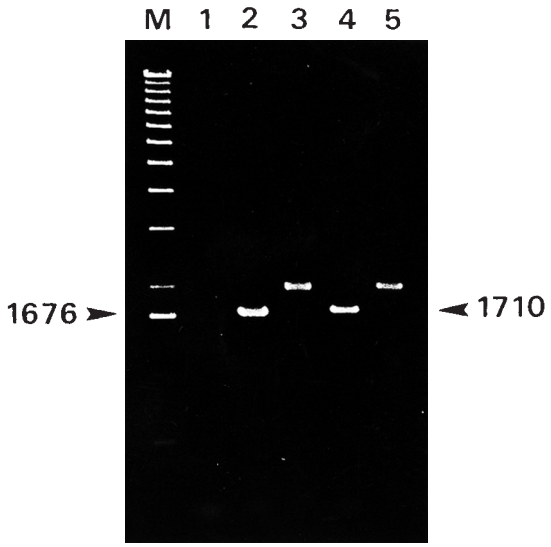

Fig. 1. Electrophoresis on 1% agarose gel of DNA products amplified with 1898/1897 primers. M = 1 kb ladder (Gibco BRL); lane 1, water; lane 2, unlabelled AS-1·7 fragment (1676 bp); lane 3, DIG-labelled 1676-bp AS-1·7 fragment; lane 4, unlabelled In-1·7 fragment (1710 bp); lane 5, DIG-labelled 1710-bp In-1·7 fragment.

Stripping and reprobing of membranes

To remove the radioactive probe, membranes were incubated in 0·2 M NaOH 0·1% (w/v) SDS three times for 20 min, at 42°C and rinsed in 2X SSC. The efficiency of the dehybridization procedure was controlled by X-ray film exposure. The membranes were used for hybridization with the DIG-labelled probe immediately, or kept wet in a plastic bag at 4°C before being rehybridized.

RESULTS

Production of '*Candidatus* Liberobacter asiaticum' and '*Candidatus* Liberobacter africanum' DIG-labelled probes

Production and DIG-UTP-labelling of the 1710-bp amplicon from '*Candidatus* Liberobacter asiaticum' (Fig. 1, lanes 4 and 5) and the 1676-bp amplicon of '*Candidatus* Liberobacter africanum' (Fig. 1, lanes 2 and 3) were controlled by gel electrophoresis. As expected, the unlabelled DNAs (lanes 2 and 4) had higher electrophotectic motilities than the DIG-labelled DNAs (lanes 3 and 5).

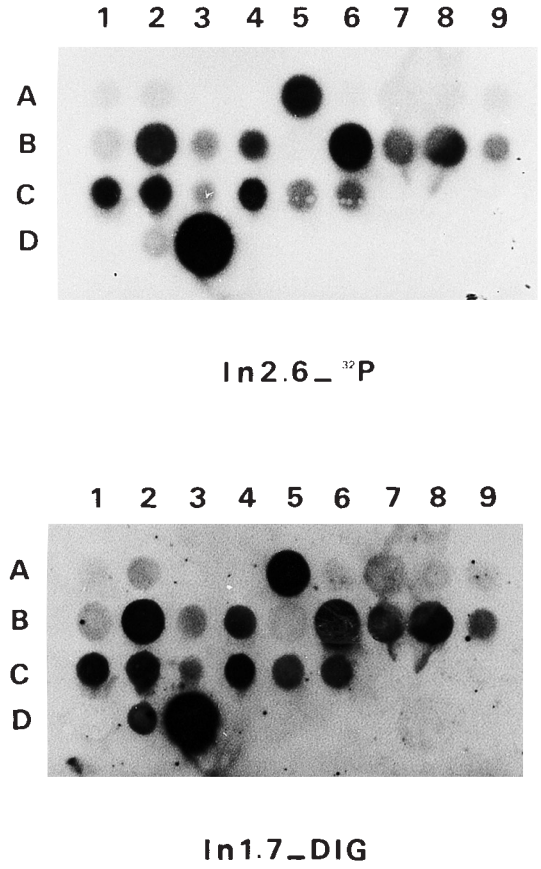

Fig. 2. Dot-blot hybridization between In-1·7-DIG or In-2·6-^{32}P probes and DNA extracted from citrus plants collected in Bali (Indonesia) (lanes A, B, C) or DNA extracted from healthy citrus plants (D1) or from citrus plants infected with Liberobacter africanum (D2) or Liberobacter asiaticum (D3) from our greenhouse.

Hybridization of radioactive and DIG-labelled In 2·6 and In 1·7 probes

^{32}P-labelled probe In 2·6 was hybridized with DNA from 24 leaf midrib samples (Fig. 2, A1 to C6) from different citrus cultivars (sweet orange, lime, mandarine) from Bali, Indonesia, where HLB is severe and widespread. Once the results had been recorded (Fig. 2, In 2·6-^{32}P) the probe was removed and the same membrane rehybridized with probe In 1·7-DIG. As expected, no hybridization signals were observed with healthy citrus DNA (Fig. 2, D1) and a strong signal was observed with DNA extracted from a greenhouse-maintained '*Candidatus* L. asiaticum'-infected sweet orange plant (Fig. 2, D3). The sensitivity of the DIG-labelled probe seemed to be slightly higher than that of the radioactive one, as sample B5 gave a positive reaction only with the DIG-labelled probe and several samples giving a faint positive reaction with the radioactive probe gave a stronger signal with the DIG-labelled probe. However, cross-hybridization with DNA extracted from '*Candidatus* L. africanum'-infected sweet orange was stronger with the DIG-labelled probe than with the ^{32}P-labelled one (Fig. 2, D2). This stronger cross-hybridization might be due to the shorter size of the DIG-labelled probe (1·7 kbp) compared to the radioactive one (2·6 kbp), but, as shown in Fig. 3 (left panel) it can be eliminated by carrying out the post hybridization stringent washing step at 65°C instead of 55°C. Indeed, the two probes gave positive hybridizations with DNA extracted from all '*Candidatus* Liberobacter asiaticum'-infected sweet orange plants from our greenhouse: China (Yunnan strain) (Fig. 3, left panel, A2), The

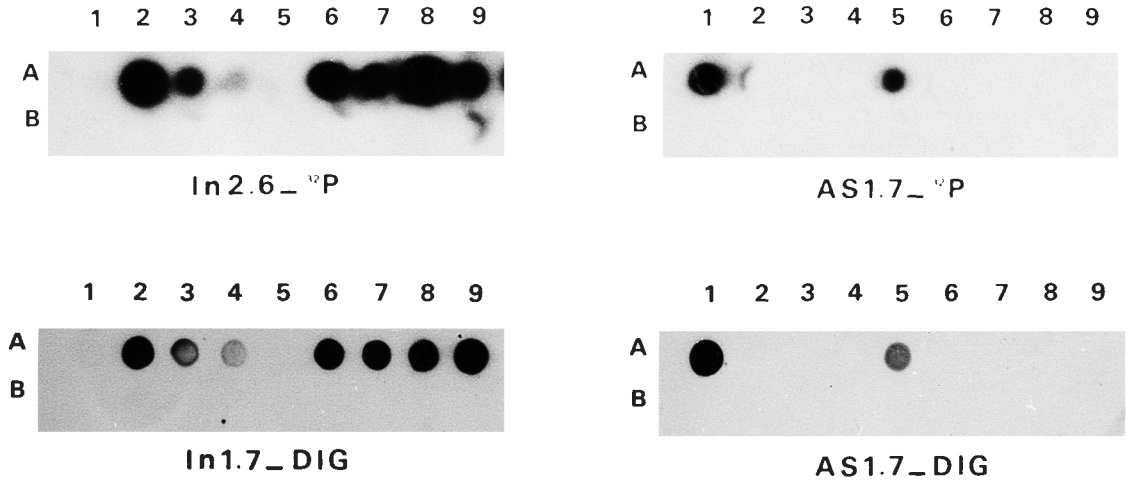

Fig. 3. Dot-blot hybridization with DIG- or ^{32}P-labelled In (left panel) or AS (right panel) probes and DNA extracted from healthy citrus plants (B6) or from citrus plants infected with the following Liberobacter strains: South Africa (A1, A5), China (Yunan) (A2), The Philippines (A3), Thailand (A4), China (Fuzhou) (A6), Taiwan (A7), India (A8, A9).

Philippines (Fig. 3, left panel, A3), Thaıland (Fig. 3, left panel, A4), China (Fuzhou strain) (Fig. 3, left panel, A6), Taıwan (Fig. 3, left panel, A7) and India (Fig. 3, left panel, A8, A9), but no hybridization with DNA extracted from healthy citrus plants (Fig. 3, left panel, B1) or from citrus plants infected with '*Candidatus* Liberobacter africanum' from South Africa (Nelspruit strain) (Fig. 3, left panel, A1, A5). However, the radioactive probe seemed to give stronger signals and we found that washing at 65°C resulted in a faint decrease of sensitivity (results not shown).

Hybridization of radioactive and DIG-labelled AS 1·7 probes

Figure 3 (right panel) shows the hybridizations of AS 1·7-^{32}P or AS 1·7-DIG with the same DNAs as those in the left panel; however the final washing step was done at 65°C. In this case, the two probes gave similar results and hybridized only with DNA of '*Candidatus* Liberobacter africanum'-infected citrus (A1, A5). No hybridization was observed with DNA extracted from healthy sweet orange plants (B1) or with DNA extracted from '*Candidatus* Liberobacter asiaticum'-infected sweet orange plants (A2 to A4; A6 to A9). When hybridization was followed by washing at 55°C, some cross-hybridization occurred with DNA from '*Candidatus* Liberobacter asiaticum' but with a similar intensity with the two probes (result not shown).

DISCUSSION

Using the previously-cloned DNA fragments In 2·6 from '*Candidatus* Liberobacter asiaticum' and AS 1·7 from '*Candidatus* Liberobacter africanum' which encode genes of the β operon, we have synthesized radioactive[4,6] and DIG-labelled probes for the detection of the two '*Candidatus* Liberobacter' species by DNA hybridization. In previous work we have used radioactive probes extensively and successfully in various countries for the detection of HLB liberobacters[10–14] Here we show that the DIG-labelled probes have a sensitivity at least equivalent to that of the radioactive probes and can therefore be used as substitute to the radioactive ones. In most HLB-affected countries, only one of the two Liberobacter species is present;[15] the two species occur together in only three areas: (i) Reunion island and (ii) Mauritius island in the Indian ocean,[13] and (iii) the border region between Saudi Arabia and Yemen. This is why for general detection purposes, and for higher sensitivity, we recommend to carry out the last stringent washing step at 55°C rather than 65°C in spite of some cross-hybridization between the two Liberobacter species at the lower temperature. However, if the two Liberobacter species have to be distinguished, for epidemiological studies for example, a stringent washing at 65°C is required.

Several countries where HLB occurs have difficulties with the use of radioelements. Thus, the availability of non-radioactive probes for the detection of the two Liberobacter species associated with HLB will be of great help. For the same reason, we have now also developed a PCR method.[16]

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