Primers based on the rpf gene region provide improved detection of Xanthomonas axonopodis pv. citri in naturally and artificially infected citrus plants

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Introduction

Xanthomonas axonopodis pv. citri (Xac) is the bacterial plant pathogen that causes citrus canker disease (Vauterin et al. 1995). Xac was formerly referred to as the ‘asiatic’ or pathotype ‘A’ form of Xanthomonas campes-tris pv. citri (Stall and Civerolo 1993). Closely related bacteria that infect either only lemons or only limes in South America were referred to as pathotypes ‘B’ and ‘C’ (Stall and Civerolo 1993), but are now considered to belong to Xanthomonas axonopodis pv. aurantifolii (Vauterin et al. 1995). Citrus canker affects the majority of the citrus production areas of the world (Goto 1992), including both Florida (Gottwald et al. 2001) and Brazil (Leite 2003). Xac is a quarantine organism and is subject to stringent international phytosanitary measures. This restricts markets for citrus from affected areas, and makes accurate identification of the pathogen critically important. Both São Paulo in Brazil and Florida in the USA support eradication programmes in

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Abstract

Aims: To have a PCR-based detection method for Xanthomonas axonopodis pv. citri (Xac) using primers designed in a specific region of its genome.

Methods and Results: A Xac-specific region was identified inside the rpf gene cluster of strain IAPAR 306 in an analysis of its complete genomic sequence. Two primers were designed, Xac01 and Xac02, which, when used in a standard PCR assay, direct the amplification of a 581 bp fragment from DNA of strains belonging to Xac from different regions around the world including unusual American and Asian strains. This product was not observed when DNA from strains of the closely related X. a. aurantifolii and X. a. citrumelo were used as templates. Extracts prepared from 28 xanthomonads of other species, and epiphytic bacteria isolated from citrus also failed to produce products with these primers. Amplification was obtained from cells grown in vitro, from extracts of both fresh and dried citrus canker lesions and from washes of inoculated but asymptomatic leaf surfaces. In sensitivity tests, this PCR technique detected as few as 100 cells.

Conclusions: Primers Xac01 and Xac02 provide specific and sensitive detection of Xac in all citrus tissues where the pathogen is found.

Significance and Impact of the Study: This PCR-based diagnostic test is suitable for monitoring asymptomatic plants in areas where the bacteria is endemic, in plant quarantine and regulatory situations, and also for obtaining an accurate diagnosis in a very short time. These are important characteristics for any assay to be used for the management of citrus canker disease.
order to protect against the serious economic consequences of this disease (Gottwald et al. 2001; Leite 2003). These programmes depend on the support of the public and legal system to facilitate the prompt elimination of disease foci and control the movement of plant material (Namekata 1991; Gottwald et al. 2001). Therefore, because of the economic and legal importance of the disease, the accurate detection of the bacteria in seedlings, budwood and asymptomatic plants, as well as in canker lesions, is critical and is also the best means to prevent further dissemination of the pathogen. Monoclonal and polyclonal antibodies have been previously raised and used for identification of Xac with generally good results. However, the antibodies may cross-react with other X. axonopodis pathogens or fail to react with newly described strains (Alvarez et al. 1991; Vernière et al. 1998).

Methods for nucleic-acid-based detection of plant pathogens have been shown to hold great promise (Henson and French 1993). Previous studies have reported primer sets specifically designed to amplify plasmid-borne genes (Hartung et al. 1993; Mavrodieva et al. 2004), virulence genes (Mavrodieva et al. 2004) or rDNA sequences (Cubero and Graham 2002) of Xac. These allowed greatly improved detection and identification of the bacterium. However, the primer sets were not entirely Xac specific. Amplification products of the rDNA sequences (Cubero and Graham 2002) of Xac. Quantitative PCR methods were also described for Xac (Cubero et al. 2001; Mavrodieva et al. 2004) based on the plasmid sequences discussed above. This technique remains expensive for use in routine diagnosis.

The genomic DNA sequences of Xac and X. campestris pv. campestris (Xcc) are quite similar, but significant differences are evident. One of these differences is in a region containing genes that regulate the expression of pathogenicity factors, the rpf genes (da Silva et al. 2002). Two rpf genes (rpfH and rpfI) from the rpf cluster present in Xcc (Dow et al. 2000) are not present in Xac, with the locus corresponding to rpfI in Xac being occupied by a truncated copy of an insertion sequence (da Silva et al. 2002). This difference prompted us to develop a new diagnostic test specific for Xac. In this paper, we demonstrate this new PCR assay using DNA purified from different species of Xanthomonas and strains of Xac from citrus canker lesions, from asymptomatic leaf surfaces of plants present in naturally infested groves, and in an artificial inoculation experiment.

Materials and methods

Analysis of the rpf region in Xac and design of the primers

A Xac-specific genomic region was identified inside the rpf gene cluster between rpfB and recJ of strain IAPAR 306 (da Silva et al. 2002) (Fig. 1). Genomic sequences in this region were used for designing primers with PrimerSelect software (DNASTAR, Inc.). The primers designated Xac01 (5'-CGC CAT CCC CAC CAC CAC GAC-3') and Xac02 (5'-AAC CGC TCA ATG CCA TCC ACT TCA-3') generate a 581 bp amplicon, and were used for these PCR tests.

Bacterial strains and culture conditions

DNA from 83 Xac strains isolated from 19 citrus-producing countries of the world, ten strains of X. a. pv. aurantifolii (the causative agent of citrus cankerosis B and C) from Argentina, Uruguay and Brazil, and one strain of X. a. citrumelo from Florida (Hartung and Civerolo 1991) (Table 1) were tested to evaluate the specificity of the primers. DNA from another 28 strains representing different species or pathovars of Xanthomonas (X. albineans, X. arboricola pv. juglandis, X. axonopodis pv. axonopodis, X. axonopodis pv. glycines, X. axonopodis pv. vasculorum, X. axonopodis pv. vesicatoria, X. axonopodis pv. vitians, X. bromi, X. campestris pv. armoraciae, X. campestris pv. campestris, X. campestris pv. mangiferaeindicace, X. campestris pv. viticola, X. cassavae, X. codiae, X. cucurbitae, X. cynarae, X. hortorum pv. hederae, X. hyacinthi, X. melonis, X. oryzae pv. oryzae, X. pisi, X. sacchari, X. theicola, X. translucens pv. graminis, X. translucens pv. translucens, X. vasicola pv. holcicola, X. vesicatoria) from different areas of the world were tested as well. These strains were...
obtained from Phytobacteria Culture Collection of the Instituto Biológico, Campinas, São Paulo, Brazil (http://www.biologico.sp.gov.br). In addition to these xanthomonads, five unidentified epiphytic bacteria with different morphologies, which were frequently isolated from canker lesions, were also tested. The strains isolated in our laboratory were grown in 3 ml of nutrient broth medium (phytone 0.5%, beef extract 0.3%, NaCl 0.1%) for 24 h on a rotary shaker (200 rev min⁻¹) at 28°C.

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* Instituto Agrônômico do Paraná, Londrina, PR, Brazil.
† Phytobacteria Culture Collection of Instituto Biológico, Campinas, Brazil.
§ Fruit Laboratory, Beltsville Agricultural Research Center, USDA.
¶ Plant Pathology Quarantine Facility, Division of Plant Industry.

DNA extractions

Two different methods were used for DNA preparation during the course of this work. Method I was used for Xanthomonas cells grown in vitro, whereas method II was used for the extraction of DNA from canker lesions on leaves and fruit of sweet orange, and also for bacterial pellets from a phosphate-buffered saline (PBS) [20 mmol l⁻¹ NaH₂PO₄/Na₂HPO₄, 0.5% NaCl (w/v), pH 6.8] wash of citrus leaves. Each is described in detail below.

Method I: Extraction of DNA from cultured bacteria

For purification of total DNA from purified Xanthomonas cells a guanidium isothiocyanate methodology was used (Pitcher et al. 1989). The DNA concentration was estimated by visual comparison with known concentrations of lambda DNA in ethidium bromide-stained 0.6% agarose gels.
**Method II: Extraction of DNA from lesions and leaf surfaces**

Citrus leaves with canker lesions and leaves on the same plant without lesions, but bordering those with lesions, were sampled. Individual canker lesions were cut with a razor blade, put in a 1-5 ml microcentrifuge tube containing 500 μl of TE buffer (50 mmol l⁻¹ Tris-HCl, pH 8·0, 5 mmol l⁻¹ EDTA, 1% (w/v) SDS) to which 0·15 mg of protease K was added. The tube was incubated at 65°C for 30 min and then the lesion was removed using a pipette tip. The DNA was further purified using the Wizard® Genomic purification kit (Promega Corporation, Madison, WI, USA), according to manufacturer’s instruction. An aliquot of 10 μl of the supernatant was used for subsequent PCR amplifications. For detection of bacteria present on the leaf surface, the leaf was cut into small pieces (∼1·5 × 1·5 mm), and put in a 50 ml tube containing 15 ml of water. The samples were shaken at room temperature for 2 h at 200 rpm. The suspension was carried to a clean tube and centrifuged at 12 000 g for 5 min to collect bacteria. The pellet was resuspended in 300 μl of TE buffer and processed with the Wizard® Genomic purification kit as above.

**PCR conditions**

PCR amplifications were conducted in volumes of 25 μl in a PTC100 thermocycler (Bio-Rad, Hercules, CA, USA). Mg²⁺ and primer concentrations as well as other conditions were optimized to amplify the DNA from Xac strain IAPAR306. The PCR reaction mixtures contained final concentrations of 2·0 mmol l⁻¹ MgCl₂, 2·5 mmol l⁻¹ (each) dNTPs (Invitrogen Corp., San Diego, CA, USA), 3·6 mmol l⁻¹ (each) primer (Xac01 and Xac02), 15 ng of template DNA from cultured cells or 10 μl of template DNA solution from leaf washes and lesions, and 1·0 U of Taq DNA polymerase (Invitrogen Corp.). An initial denaturation step at 94°C for 3 min was followed by 36 cycles of: denaturation at 94°C for 45 s, annealing at 60°C for 45 s and elongation at 72°C for 45 s, followed by a final extension incubation of 5 min at 72°C. All reactions were prepared using filter-barrier pipette tips. Aliquots of 10 μl from amplification reactions were subjected to electrophoresis through 1·0% (w/v) agarose gels. The gels were stained with ethidium bromide and examined under ultraviolet light.

**Infestation of leaves and estimation of the limits of detection of the PCR test**

To determine the lower limit of PCR detection, cells of strain IAPAR306 were grown for 24 h in nutrient broth at 28°C and 200 rpm to a concentration of 1 × 10⁸ cells ml⁻¹, estimated using a Neubauer chamber. A tenfold dilution series was prepared to the ninth dilution in PBS. Buffer was used as a no template control. For a simple estimation of detection limits using the bacterial suspension, the tenfold dilution series tubes were centrifuged at 12 000 g for 5 min, the supernatant was discarded and the pellet was resuspended in 300 μl of TE buffer and processed with the Wizard® Genomic purification kit. For estimation of the sensitivity of the method when sampling leaf surfaces, tubes containing bacterial suspensions were centrifuged at 12 000 g for 5 min and the supernatant was discarded. The pellet was resuspended in 200 μl of Milli-Q water and dropped over the entire surface of a fully expanded citrus leaf. The infested leaf was dried at room temperature and immediately processed for PCR analysis as described for method II.

**Results**

**Unique aspects of the rpf cluster region**

Computer analysis showed that the rpf cluster in Xac is different from the rpf cluster present in Xcc (Fig. 1). In Xcc orf3 is adjacent to rpfJ, which is absent in Xac. In its place, there is a 6 kb region carrying ORFs of unknown function. On one side of the cluster there is a wapA (wall associated protein) homologue that is physically linked to a gene present in both bacteria, recJ. This unique aspect of the Xac rpf gene cluster prompted us to evaluate the presence of this structure in other xanthomonads. To do this we decided to amplify a region comprising the 5’ end of the wapA homologue and the 3’ end of recJ (Fig. 1). For strain IAPAR306 of Xac, the reactions were expected to generate fragments of 581 bp.

**Specificity of the PCR**

The predicted amplification product (581 bp) was obtained for all isolates of Xac from different regions around the world, including the genetic variants A⁷ and A⁸, which occur in southwestern Asia and southern Florida, respectively. No amplification product was obtained with X. a. aurantifolii, the causative agent of cancrosis ‘B’ and ‘C’, or with X. a. citrullina, the causal agent of citrus bacterial spot in Florida nurseries (Fig. 2). DNA from 28 additional non-Xac xanthomonads was used as template for PCR amplification. No amplification product was observed from any non-Xac strains, except for one strain of X. alboocinensis, where a 400 bp fragment was amplified, an amplification product of substantially different size compared to the specific fragment obtained for Xac (581 bp). Five uncharacterized bacteria isolated from
Amplification of Xac from canker lesions

Extracts of canker lesions from 'Natal', 'Pera' and 'Valencia' sweet orange varieties field-grown in infected areas showed the expected amplification product, as did asymptomatic leaves bordering diseased leaves (Fig. 3). Amplification products were not observed when extracts of healthy leaves obtained from distant nonsymptomatic plants were used as templates or in reactions without a DNA template (Fig. 3).

Sensitivity of PCR

The sensitivity of the amplification assay for Xac was determined using a tenfold dilution series of cells of Xac strain IAPAR306. The PCR allowed us to detect as few as 100 cultured Xac cells using DNA extracts prepared directly from the tenfold dilution series (Fig. 4a). The same result was obtained starting with leaves that had the bacteria spread on their surface (Fig. 4b).

Discussion

In this paper, we describe a PCR-based assay for Xac detection. DNA sequence comparison of the rpf region of Xac and Xcc (da Silva et al. 2002) allowed us to develop a strategy for the detection of Xac to the exclusion of all other xanthomonads and related species and strains tested. The basis for this Xac-specific PCR assay is the replacement, in Xac, of rpfJ with a 71-bp region containing two transposases, two hypothetical proteins and a wap homologue (Fig. 1). Unlike previously described primers (Hartung et al. 1993; Mavrodieva et al. 2004), this assay is based on a genomic DNA sequence. We designed primers for this region, which amplify a 581 bp fragment specific to Xac. These primers flank the region between the wapA homologue (primer Xac02) and recJ (primer Xac01) (Fig. 1). The high specificity of these primers was because of the Xac02 primer designed within the Xac-specific wapA homologue insert (Fig. 1). This is because recJ, the site of primer Xac01, is conserved, with 84% identity at the nucleotide level between Xac and Xcc. The specificity of the primers was confirmed by the amplification of the expected fragment (581 bp) in all isolates of Xac from countries located in different regions around the world (Australia, Guam, Maldives Islands, Mauritius, New Zealand, Reunion, Saudi Arabia and Yemen) where citrus is grown, including the Americas and Asia. Unusual genetic variants of Xac type A found in Florida, causing citrus canker on Key/Mexican lime (C. aurantifolia) (A\textsuperscript{W}), and in southwest Asia (A\textsuperscript{*}) (Vernière et al. 1998; Sun et al. 2004) were also successfully amplified. The amplification of the targeted region in A\textsuperscript{*} and A\textsuperscript{W} variants demonstrates the presence of this region in all strains of Xac. Further, the absence of amplification in all other xanthomonads, including X. axonopodis pv. auran
tifolii, demonstrates the high degree of specificity of the designed primers. No amplification was observed with...
epiphytic bacteria associated with canker lesions. The specificity of the primers may also have evolutionary significance as it seems that the genomic organization of the rpf region is unique for Xac compared to other xanthomonads, suggesting that the insertion of the sequence in the middle of the cluster is a recent evolutionary event. PCR amplification was successfully used to detect cells of Xac in citrus canker lesions from different varieties of sweet orange. Further, Xac was also detected on adjacent asymptomatic leaves (Fig. 3). Uncharacterized components of plant tissues often inhibit the amplification of nucleic acids by PCR (Wilson 1997). The DNA purification strategy described in this paper was essential to successfully amplify the PCR product from extracts of older canker lesions. Inconsistent results were obtained when using unpurified lesion extracts or leaf washes directly as the source of template DNA (data not shown). No amplification was observed when the Xac concentration was below 100 cells ml\(^{-1}\). Previous reports have indicated that the minimum numbers of Xac cells required to induce citrus canker lesions through infection of wounds and stomata ranged from 10\(^2\) to 10\(^3\) and from 10\(^4\) to 10\(^5\) cells ml\(^{-1}\), respectively (Zubrzycki and Diamante 1987). Xac cell numbers on asymptomatic leaves can range from <10\(^1\) to as high as 10\(^5\) CFU per leaf (Stall and 1987). Xac cell numbers on asymptomatic leaves can

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