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In planta-polymerase-chain-reaction detection of the wilt-inducing pathotype of *Fusarium oxysporum* f.sp. *ciceris* in chickpea (*Cicer arietinum* L.)

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A 1.6 kb fragment of random amplified polymorphic DNA (RAPD-PCR, polymerase chain reaction), which was specific for race 5, a wilt-inducing isolate of *Fusarium oxysporum* f.sp. *ciceris* (*Foc*), was cloned and sequenced. This fragment was not detected in RAPD-PCR reactions with DNA from yellowing-inducing pathotypes of *Foc*, or from other fungi tested. Specific PCR primers were designed from the sequence data and used to detect the presence of the fungus in genomic DNA isolated from symptomless chickpea plants, 16 days after inoculation. A single, 1.5 kb PCR product was only observed in PCR reactions with DNA from plants infected with a wilt-inducing isolate. No products were observed in reactions with DNA from plants infected with yellowing-inducing pathotypes, or from DNA isolated from uninfected chickpea cultivar controls. Southern hybridization demonstrated homology between the second PCR product and the original specific wilt-associated RAPD fragment. PCR products were detected with DNA extracted from roots and stem tissue, but no fungal DNA was detected in leaf tissue of the same infected plants. In a blind trial, the specific primers correctly identified the fungal pathotype in four different, wilt-infected chickpea cultivars.

INTRODUCTION

Fusarium wilt of chickpea, caused by *Fusarium oxysporum* Schlect.: Fr. f. sp. *ciceris* (Padwick) Matuo & Sato (*Foc*), was first reported in India [35] causing a rapid flaccidity, desiccation and progressive yellowing of infected plants. The fungus has since been reported from all areas of chickpea cultivation, including Bangladesh, Burma, California, Ethiopia, India, Malawi, Mexico, Morocco, Pakistan, Peru, Syria, Tunisia, Turkey, USSR and Spain [16, 23, 33, 42, 44]. Seven races of *Foc* have been characterized by testing the resistance or susceptibility of ten host cultivars (cvs) to the pathogen [18, 22]. Analysis of Spanish races of *Foc* revealed the existence of two

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Abbreviations used in text: *Foc*, *Fusarium oxysporum* f.sp. *ciceris*; RAPD-PCR, random amplified polymorphic DNA-polymerase chain reaction; SCARs, sequence-characterized-amplified regions.

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different pathotypes, causing yellowing or wilting of infected plants. Races 2, 3, 4, 5 and 6 induced the wilt syndrome, severe chlorosis and flaccidity together with vascular discolouration and early plant death by 20 days after inoculation. In contrast, isolates of race 0 induced the yellowing syndrome, progressive, foliar chlorosis with vascular discolouration, and later death 40 days after inoculation. Biological pathotyping for the identification of the different races and pathotypes of Foc takes at least 40 days to complete, and given the economic importance of this pathogen, there is a requirement for rapid diagnostic tests. Consequently, different molecular methods have been assessed for their suitability in distinguishing Foc isolates [24]. Randomly amplified polymorphic DNA fragments (RAPDs) [43, 47] are useful in identifying variation and pathotypes in a wide variety of fungi [1, 12, 15], including several different formae speciales of F. oxysporum [2, 3, 4, 6, 13, 28]. We have identified several RAPD fragments which discriminate between wilt- or yellowing-inducing pathotypes of Foc [24, 25]. One of these, a 1.6 kb DNA fragment, generated with a single primer KS, was shown to be specific for the wilt-inducing pathotypes. The polymerase chain reaction (PCR) has been successfully used for the detection of fungal DNA in infected plants [19, 20] and sequence characterized amplified regions (SCARs) have been used for the specific amplification of the target DNA [29, 36]. The objective of this study was to sequence the wilt-specific fragment and to use this to design PCR primers for the detection of wilt-specific fungal DNA in infected plants.

MATERIALS AND METHODS

Chemicals and media

All media were made up with glass distilled water and autoclaved at 121 °C for 15 min. All reagents (analytical or molecular biology grade) were from BDH Chemicals Ltd. or Sigma Chemical Co. unless otherwise stated. DNA restriction and modification enzymes were from Promega. The *Escherichia coli* strain used in cloning experiments was XL1-BlueTM and the vector was pBluescriptTM II KS+ (Stratagene).

Fungal strains and growth conditions

Isolate 8012 (a wilt-inducing isolate of race 5), and isolates 7802, 7952 and 9018 (yellowing-inducing isolates of race 0) of *Fusarium oxysporum* f.sp. ciceris (Foc) were cultured and maintained as described [22]. In addition to these isolates, DNA from a total of 28 wilt-inducing and 34 yellowing-inducing isolates of Foc, as described by Kelly et al., [24, 25] was also analysed. Other fungi used were; six isolates of *F. oxysporum* f.sp. niveum (one from Cyprus and one from Spain), two isolates of *F. oslani*, *F. eumartii*, Didymella (Ascochyta) rabiei (all from Spain) [24, 25], Verticillium dahliae and V. albo-atrum. Fungal mycelium for the extraction of DNA was produced as described [25].

Chickpea cultivars

Chickpea cvs PV13, PV60, CPS1 and ICCV2 were germinated, inoculated with cultures of the four isolates of *Foc*, and grown, with uninfected control plants, for 16

DNA extraction

Fungal DNA was extracted from fungal biomass and analysed as described previously [25]. Plant genomic DNA or total genomic DNA from fungal-infected plants was extracted using a cetyl-trimethylammonium bromide (CTAB) method [9, 31]. Plant material, uninfected or infected, was freeze-dried and ground to a fine powder in a sterile pestle and mortar. A sample of 50 mg of this powder was transferred to a sterile 1.5 ml microcentrifuge tube, and to this was added 900 µl of prewarmed (60 °C) CTAB extraction buffer [100 mм Tris pH 7·5, 1% w/v CTAB, 0·7 м NaCl, 10 mм EDTA, $1 \frac{0}{10}$ v/v 2-mercaptoethanol (added just prior to use)]. Incubation at 60 °C for 30 min with occasional agitation was followed by the addition of 450 µl of chloroform/isoamyl alcohol (24:1 v/v). The tubes were mixed by inversion for 5 min and centrifuged for 10 min at 11400 g in a microcentrifuge (MSE, Microcentaur). The upper aqueous phase was carefully transferred to a fresh tube and 0.1 volume of prewarmed (60 °C) 10 % CTAB in 0.7 м NaCl was added and mixed thoroughly. The chloroform extraction and centrifugation were repeated and the aqueous phase transferred to a fresh tube. An equal volume of precipitation buffer (50 mM Tris pH 8.0, 10 mM EDTA, 1% w/v CTAB) was added. Following inversion, the tubes were left at room temperature for 30 min to allow the DNA to precipitate. The DNA was pelleted as before, the supernatant removed and the pellet dissolved in 450 µl of 1 M NaCl. The DNA was re-precipitated by the addition of 900 μ l of 100 % ethanol. The pellet was washed in 70% ethanol and dissolved in 100 µl of TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8·3). DNA samples were stored at -20 °C. The concentration and purity of DNA samples were determined by measuring absorbance at 260 and 280 nm using a Hewlett-Packard HP8452A Diode Array Spectrophotometer. DNA size was determined by agarose electrophoresis against known standards (1 kb DNA markers, Gibco-BRL).

Cloning and sequencing of the wilt-associated RAPD marker

Standard molecular biological methods were used [38] unless otherwise stated. DNA fragments and PCR products were analysed on 0.9 % or 2% horizontal agarose gels (MP agarose, Boehringer Mannheim, Germany), in 0.5X TBE (45 mM tris-borate, 1 mM EDTA) or TAE (40 mM tris-acetate, 1 mM EDTA). Gels were run at 5 V cm⁻¹ for 2–3 h. Gels were stained in $0.5 \mu g$ ml⁻¹ ethidium bromide solution for 30 min prior to being photographed on Polaroid 665 or 667 film under ultra-violet illumination. The 1.6 kb wilt-associated band, generated by RAPD-PCR with the single primer KS (Stratagene, 5'-CGAGGTCGACGGTATCG-3') from the race 5 isolate 8012 of *Foc*, was recovered from agarose gels, as described [24]. The DNA was purified using MagicTM clean-up columns (Promega) according to the manufacturers' instructions,

ligated into *SmaI* digested plasmid pBluescriptTM II KS + (Stratagene), and used to transform competent XL1-BlueTM cells [5]. Recombinant XL1-BlueTM cells were detected by blue/white screening on ampicillin/tetracyline/IPTG/X-gal Luria-Bertani plates, according to the manufacturers' instructions. White colonies were subsequently screened for insert size. Plasmid DNA was isolated from 3 ml of overnight cultures of recombinant clones using MagicTM MiniPreps (Promega), according to the manufacturers' instructions.

Restriction maps were made of those clones containing an insert of the correct size, and these were analysed by labelling and hybridization back to the original Southern blots of RAPDs [24]. Three different recombinant pBluescript[™] II KS+ clones containing the 1.6 kb RAPD insert DNA were sequenced by the dideoxy method [39], using a Sequenase[®] 2.0 kit (USB Research, Amersham), according to the manufacturers' instructions. The sequencing products were analysed on 8 % denaturing polyacrylamide gels (PAGE, National Diagnostics) using a 2010 Macrophor sequencing system (Pharmacia Biotech). The PAGE gels were transferred to 3 MM paper and dried under vacuum at 80 °C for 30 min. Autoradiographs were made using Fuji RX X-ray film or Amersham Hyperpaper at room temperature with exposure times of 12-48 h. Additional ABI automatic sequencing was performed by the Department of Molecular Medicine, King's College London School of Medicine and Dentistry. Sequences were analysed and compared using the University of Wisconsin's Genetic Computer Group (GCG) sequence analysis package 7.0 [7]. From the resulting 1618 bp sequence, specific PCR primers, wilt-1 (5'-TGATGTGAGGACGGCCAGG-3', complementary sequence to nucleotides 1539–1557) and Wilt-2 (5'-TATCAGA-GCATCTCCCTCCC-3', corresponding to nucleotides 94–113), were designed using the PRIMER routine of GCG, and used to amplify a 1464 bp portion of the KS 1.6 kb RAPD fragment (KS16).

Labelling of probe DNA, Southern Blotting, and hybridization of transferred DNA

The 1.6 kb wilt-associated RAPD DNA fragment was labelled using DIG-11-dUTP (digoxigenin-3-O-methylcarbonyl-(-amino-caproyl-5-(3-aminoallyl)-uridine-5'-triphosphate) according to the manufacturers' instructions (Specific PCR Reactions, Boehringer-Mannheim, Germany). Fungal DNA, and DNA from infected and non-infected plants were analysed by electrophoresis and transferred [41] onto Hybond N membranes (Amersham International) using a vacuum transfer system (VacuGene XL, Pharmacia Biotech). Identification of homologous sequences of DNA, using a labelled probe, was by the DIG-detection chemiluminescent method (Boehringer-Mannheim), and signals were recorded on either Fuji RX X-ray film or Amersham Hyperfilm, following the manufacturers' instructions.

Specific PCR reactions

The PCR oligonucleotide primers wilt-1 and wilt-2 were synthesized by the Molecular Biology Unit at King's College, London. PCR reactions were performed in thermal cyclers TR1, TR2, or Omni-Gene (Hybaid Ltd.). The 100 µl reaction mixtures consisted of 100 ng–1 µg of genomic DNA extracted from fungal material, or from

infected or uninfected chickpea material, 200 μ M of each deoxynucleotide-triphosphate, 0·5 μ M of each primer, 1·5 mM MgCl₂, reaction buffer (50 mM KCl, 10 mM Tris-HCl pH 9·0 [25 °C], 1 % v/v Triton[®] X-100, Promega) and 2 Units of Taq DNA polymerase (Promega). Samples were overlaid with 25 μ l of filter-sterilized light mineral oil (Sigma). A hot-start procedure [*11*] in the absence of Taq DNA polymerase was employed in PCR reactions. The samples were heated to 94 °C for 5 min to fully denature the DNA, and subsequently cooled to 72 °C to allow addition of the Taq DNA polymerase. The samples were then subjected to 40 cycles of 1 min of primer annealing at 58 °C, 3 min extension at 72 °C, 1 min denaturation at 94 °C, followed by a final annealing step of 1 min at 58 °C and a final extension step of 5 min at 72 °C. Twenty microlitre samples were analysed by agarose gel electrophoresis as described above.

RESULTS

Sequencing of the 1.6 kb wilt-associated RAPD marker KS16

The RAPD fragment KS16 was amplified, cloned and sequenced (Fig. 1). The sequence has been deposited in the GenBank database, accession no. AF005633. The data obtained showed the presence of the initial RAPD primers used (KS) at both ends of the insert DNA. Analysis of the six possible open reading frames (ORFs) showed the presence of numerous stop codons in all orientations. A BLAST search of the GenEmbl data base showed no significant similarity with any DNA sequence. No fungal regulatory motifs, such as enhancers, promoters or terminators could be detected [14], and there were no significant repetitive regions. A putative initiation codon was present at 921 bp and a possible intron between 1115 and 1194 bps. When the intron was removed and the remaining sequence was translated, a possible 74 amino acid residue peptide could be identified. This was compared with the SWIS-PROT data base but again no significant similarity with other peptides could be detected. The RAPD fragment was labelled and hybridized to Southern transfers of restricted genomic DNA and autoradiographs obtained showed multiple bands (data not shown).

PCR of fungal DNA from pure cultures and DNA from infected chickpea using specific primers Fungal DNA was extracted from pure cultures of wilt-inducing and yellowinginducing isolates. The primers wilt-1 and wilt-2 were used to amplify a 1.5 kb band from this DNA and from the DNA previously isolated from a range of fungal isolates (see Materials and Methods). There was a complete association of the 1.5 kb fragment with wilt-inducing isolates (28 isolates tested). The band was absent in all yellowinginducing isolates (34 isolates tested), and absent from reactions with all other fungi tested. These included two other formae speciales, two other species of *Fusarium*, other chickpea fungal pathogens (*Didymella = Ascochyta*) and two species of *Verticillium* [24]. Specific PCR was performed upon chickpea DNA, using the same *Foc* wilt-associated specific primers, to determine whether fungal DNA could be amplified in an excess of chickpea DNA. DNA was extracted from the following samples: root and stem tissue from four healthy, uninoculated chickpea cvs. (PV13, PV60, CPS 1 & ICCV 2),

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KS1

	CGAGGTCGAC	<u>GGTATCG</u> GCT	ACATAATAAG	GCGCAATGGC	TTCAATGCTT	CGTCTTCCCG
				wilt-2		
61	CCAAACCAAG	GCTTCCGATC	TTAAGAGAAC	TTC <u>TATCAGA</u>	GCATCTCCCT	<u>CCC</u> ACCCGAC
121	TGACGTGCTC	CCTATCTCGC	GATATAGCGC	TGACTCTATT	TTACTCCCTC	TCGATAAATT
181	GGAGGAGATT	GGGCCTTTAT	TTAGTCTTTA	CGAGAATATA	TGATGGAGAG	AATAGCACTT
241	GAGAGTGAGG	GTATTAAAAA	TATCGGTAGC	ACGTCGCCAC	AGACCGCTTG	AGATTCACGA
301	TIGGTCTACT	AGATTGCTCA	GCCGGCGAGC	TGACATATTT	TCTTTTTCCG	ACATAAGGGT
361	ACGTTTCGGG	AAAATCTGCT	GCGACAACAT	CAACCCCAAT	AATGGCCCCA	TAGCCAAGCC
421	GACCCTCACC	татстстста	TGGCCGGTTG	TATGGCGTTG	GAGAGGGATA	TTTAAACCGG
481	CGAATAACTA	GAAGTCATTG	TTAGACAAGG	CCTCATCGAG	ATCACAGGTA	AACTGTAGAT
541	GCCATAATAA	GTATTAGCAG	TTCTTCTAGT	атаастаата	GTAGATTTAA	TGCTGGTATT
601	GCTCTAGAAA	GGAAAGCATA	GTGACACCTA	GGACCAACTT	CGGCAGTIGG	CATCATCCGC
661	GTAATTAACC	CCTGCCGCTT	TGGGGCACGC	AATTTTAACC	ATCTGTTTCA	TTGCACGTCC
721	GGGCTTGTTC	ATTGTTAACG	CAAAAGCAAA	TAAAGCATGT	ATGAAAACTT	ATGCTACGGT
781	GTCACATACA	CGGTCAATAA	CAACCAATCA	ACCGACCATA	CGTGTTCTCT	CTACTAGCTA
841	GCTTTCATCA	TATAGCCAAA	ACATAGTCAT	CGCGCATAAC	CATCATGTTT	TCGCAGTGAG
901	AGAGACTTTG	CATACCCCAT	TATGGGTCCC	CGCGCACTCT	CGGCACAGGA	GAAAGCGGAC
961	AGGCCTGTTC	GTGAGCGAAA	CAGGGGAAGC	CAACCCCGCC	TGGATGCCGC	CCAAAACGGC
1021	CGGACCCACC	GTCCCGATTC	CGATCTGAAC	AACACAAATG	ATATTCTAGA	CATTCCCACG
1081	AGCAGTTTAT	CTACAGAAAT	AATTACGGGA	AGAAGTACAG	TTCTTGGTAA	ATATCCCTAC
1141	TTGTTTCAAA	TTCCGCTTTA	ACTCGCAGGA	CTAAGCAGTG	ATGTCTACCG	ACAGATAACG
1201	GAAACCAGTG	CGACGGTTTT	ACCCTAGACC	TGATTGACGA	ACAACGACGA	ACCIGCGIGA
1261	CTCCTCGAAG	AAGCCAGACC	CCACCCGCTA	ATCAATCGAT	CGTAACGACG	CACGAGCCGA
1321	TAGTACAAGT	GGATGAAACA	GCCCTGCGAC	CGGGTATCTC	CACACTGTCG	ATCGACGAGC
1381	ATACATACCC	CAATCCTCCT	TGTATCGCAG	CACAATGTCC	TGTGATCACG	AACCCTCAGG
1441	ATATGGCGAT	TGTAGGGGAT	GAGGCGGAGC	TTATCTCTAC	AGTTGAAGTC	TCAGGCGCGA
1501	CATTGACCAC	AAAAGATGAG	CAGCAGGCTA	CTCCTAAA <u>CC</u>	TGGCCGTCCT	wilt-1 <u>CACATCA</u> ATC
1561	CCGCCGCATC	ACCGCGTGAG	CGAACGCGTC	TCCGCGTCCA	A <u>CGATACCGT</u>	CGACCTCG

KS2

FIG. 1. For legend see opposite.

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FIG. 2. Detection of *Fusarium oxysporum* f. sp. *ciceris* DNA by specific PCR of DNA from root, leaf and stem tissue of symptomless infected chickpea cv. PV13, using wilt-associated specific primers wilt1 and 2. DNA was fractionated on a 2% agarose gel and stained with ethidium bromide. Numbers on the side are in kb pairs. The lanes were : (1) 1 kb DNA ladder (Gibco-BRL); (2–5) leaf DNA; (6–9) root DNA; (10–13) stem DNA; (14) uninfected-root DNA (PV13); (15) control (no DNA). Amounts of DNA: 2, 6, 10 and 14 = 1 μ g; 3, 7 and 11 = 0.5 μ g; 4, 8 and 12 = 0.1 μ g; 5, 9 and 13 = 0.05 μ g.

together with the corresponding infected, but symptomless, tissue from chickpea cvs. inoculated separately with each of the four different isolates of Foc (7802, 7952, 9018 and 8012). In addition, six infected cultivars (A-F), where the identity of the Foc isolate was unknown to the King's College researchers, were used in a blind trial. Initial reactions were performed to determine the amount of chickpea DNA required in order to detect fungal DNA in infected host cvs. DNA was extracted from the roots, stems and leaves of infected, but symptomless, chickpea cv. PV13, inoculated with Foc race 5 isolate 8012. Differing amounts of DNA from each of the tissues were employed in a PCR reaction using the wilt-associated specific primers wilt 1/2. A 1.5 kb band corresponding to the wilt-associated RAPD marker was observed for both root and stem DNA, but not from leaf DNA (Fig. 2). Fungal DNA was detected from 100 ng of DNA from root and stem tissue of *Foc*-infected chickpea but not from the leaf (Fig. 2). When the primers wilt-1 & 2 were used in specific PCR reactions with DNA from chickpea cvs. tissue samples from plants infected with the Foc wilt-inducing isolate 8012 (race 5), a 1.5 kb band was observed with all combinations except for cv. CPS 1 stem and cv. ICCV 2 stem (Fig. 3, Top gel, lanes 15 and 17) (Fig. 3). These two negative tissue samples were later shown to contain the relevant band by hybridization to the specific probe (see below). The same band was also observed with the blind trial samples A (root and stem), B (root), D (root and stem) and E (root) (Fig. 3; Bottom gel, lanes 16, 17, 18, 22, 23 and 24).

FIG. 1. Sequence of plasmid pKS16 insert DNA (KS16, 5'-3'). The positions of the primer KS sequences and the specific primers wilt-1 (complementary sequence) and wilt-2 are underlined. The vector sequence is not included. The GenBank Accession number is AF005633. The deposited sequence excludes the KS primer sequences from the 5' and 3' ends.



FIG. 3. Specific PCR of Fusarium oxysporum f.sp. ciceris DNA from root and stem tissue of uninfected, and asymptomatic, infected chickpea plants using wilt-associated primers wilt1 and wilt2. DNA was fractionated on a 2% agarose gel and stained with ethidium bromide. Numbers on the side are in kb pairs. Both gels: lane 1, 1 kb ladder (Gibco-BRL). (a) Top gel. Uninfected tissue (controls), lanes as follows: (2) PV60 root; (3) PV60 stem; (4) CPS 1 root; (5) CPS 1 stem; (6) ICCV 2 root; (7) ICCV 2 stem; (8) PV13 root; (9) PV13 stem. Infected tissue: (10) PV13/8012 root; (11) PV13/8012 stem; (12) PV60/8012 root; (13) PV60/8012 stem; (14) CPS 1/8012 root; (15) CPS 1/8012 stem; (16) ICCV 2/8012 root; (17) ICCV 2/8012 stem; (18) CPS 1/9018 root; (19) CPS 1/9018 stem; (20) CPS 1/7802 root; (21) CPS 1/7802 stem; (22) CPS 1/7952 root; (23) CPS 1/7952 stem; (24) PV60/7952 root; (25) PV60/7952 stem; (26) PV60/9018 root; (27) PV60/9018 stem. (b) Bottom gel. Infected tissue, lanes as follows: (2) PV60/7802 root; (3) PV60/7802 stem; (4) PV13/9018 root; (5) PV13/9018 stem; (6) PV13/7802 root; (7) PV13/7802 stem; (8) PV13/7952 root; (9) PV13/7952 stem; (10) ICCV 2/9018 root; (11) ICCV 2/9018 stem; (12) ICCV 2/7952 root; (13) ICCV 2/7952 stem; (14) ICCV 2/7802 root; (15) ICCV 2/7802 stem. Blind trial samples: (16) A root; (17) A stem; (18) B root; (19) B stem; (20) C root; (21) C stem; (22) D root; (23) D stem; (24) E root; (25) E stem; (26) F root; (27) F stem.

Southern blotting and hybridization

Southern blotting of the specific PCR gel, and hybridization with labelled insert DNA from pKS16, showed that the PCR products (Fig. 3) were homologous to the original wilt-specific RAPD-PCR marker (Fig. 4). No signals were observed in lanes corresponding to cvs. infected with yellowing-inducing isolates of *Foc*, or in lanes corresponding to uninfected cvs, although there was some hybridization of the probe to unincorporated primers, and to DNA which was retained in the loading wells. In addition, homologous bands of the correct size were also seen in the lanes corresponding to the two tissue samples which appeared to have no amplification products in Figure 3 (see above: Fig. 4, top gel, lanes 15 and 17). In addition, signals of the correct size were also seen in the lanes B (stem) and E (stem) (Fig. 4; bottom gel, lanes 19 and 25), although no specific PCR products could be seen in the original gel (Fig. 3; bottom gel, lanes 19 and 25).



FIG. 4. Southern transfer of specific PCR products, using wilt-associated primers wilt1 and wilt 2, from genomic DNA extracted from root or stem tissue of uninfected, and infected but symptomless chickpea plants. The transfer was probed with labelled KS16 DNA. DNA was separated on a $2\,\%$ agarose gel and stained with ethidium bromide. Numbers on the side are sizes in kb pairs. Both gels: lane 1, 1 kb ladder (Gibco-BRL). (a) Top gel. Uninfected Tissue, lanes as follows: (2) PV60 root; (3) PV60 stem; (4) CPS 1 root; (5) CPS 1 stem; (6) ICCV 2 root; (7) ICCV 2 stem; (8) PV13 root; (9) PV13 stem. Infected tissue: (10) PV13/8012 root; (11) PV13/8012 stem; (12) PV60/8012 root; (13) PV60/8012 stem; (14) CPS 1/8012 root; (15) CPS 1/8012 stem; (16) ICCV 2/8012 root; (17) ICCV 2/8012 stem; (18) CPS 1/9018 root; (19) CPS 1/9018 stem; (20) CPS 1/7802 root; (21) CPS 1/7802 stem; (22) CPS 1/7952 root; (23) CPS 1/7952 stem; (24) PV60/7952 root; (25) PV60/7952 stem; (26) PV60/9018 root; (27) PV60/9018 stem. (b) Bottom gel. Infected tissue, lanes as follows: (2) PV60/7802 root; (3) PV60/7802 stem; (4) PV13/9018 root; (5) PV13/9018 stem; (6) PV13/7802 root; (7) PV13/7802 stem; (8) PV13/7952 root; (9) PV13/7952 stem; (10) ICCV 2/9018 root; (11) ICCV 2/9018 stem; (12) ICCV 2/7952 root; (13) ICCV 2/7952 stem; (14) ICCV 2/7802 root; (15) ICCV 2/7802 stem. Blind trial samples: (16) A root; (17) A stem; (18) B root; (19) B stem; (20) C root; (21) C stem; (22) D root; (23) D stem; (24) E root; (25) E stem; (26) F root; (27) F stem.

DISCUSSION

The wilt pathotype-specific RAPD-PCR band was cloned, and sequenced. Use of the labelled 1.6 kb band as a probe showed that it had homology to itself but not to other RAPD bands generated using the same primer. No hybridization was observed to fungal genomic DNA from yellowing-inducing isolates. GCG sequence analysis software provided no evidence of homology with other sequences. A putative open reading frame also showed no homology with protein data bases, and other lines of evidence suggested that it was a repetitive non-coding sequence. The production of multiple bands in genomic DNA Southern transfers, and hybridization to more than one chromosome in CHEF gels (Kelly, unpublished data) supported the conclusion that the fragment was a non-coding, dispersed, repetitive sequence similar to that found in F. axysporum niveum [26]. Alternatively, the sequence could be associated with

a transposon or retrotransposon [27, 30]. However, no similarity was detected with fungal transposons in the databases. Repetitive DNA sequences of unknown function have been used to identify sub-populations within fungal species successfully, and to demonstrate pathogenic specialization in a number of fungi such as *Magnaporthe grisea* [17], *F. oxysporum* f.sp. *pisi* [45] crucifer-infecting *F. oxysporum* [27], cucurbit-infecting *F. oxysporum* [32] and non-pathogenic *F. oxysporum* [10]. In *Cladosporium fulvum*, all but one of 49 RAPD markers examined consisted of repetitive DNA which, given the high level of repetitive DNA in phytopathogenic fungi, might not be unexpected [1]. This, however, does not exclude their use in distinguishing between fungi, and identifying loci linked to genes of interest.

PCR primers were designed from the sequence of the cloned RAPD marker and used to amplify a 1.5 kb portion of this fragment. These primers, wilt-1 and 2, generated a 1.5 kb product with all the known wilt-inducing isolates tested and no band was detected with yellowing-inducing isolates or with the other fungi tested. In addition, the primers detected the presence of wilt-inducing DNA in infected but symptomless plants. Four blind trial cvs were also correctly identified as being infected with wiltinducing isolates of *Foc*, the other two cvs being infected with yellowing-inducing isolates. The use of primers derived from sequences of RAPD markers was first described in plant pathology by Paran and Michelmore, who used these sequence characterized amplification regions (SCARs) to identify plant sequences linked to downy mildew resistance genes in lettuce [*36*]. SCARs have been shown to be extremely useful in detecting, *in planta*, the presence or absence of the blue mould pathogen *Peronospora tabacina* in tobacco [*46*], *F. culmorum*, *F. graminearum* and *F. avenaceum* [40] and *Erysiphe graminis* in barley [29].

The primers could detect fungal DNA in the roots and stems of chickpea cvs in symptomless infected plants. The wilt-specific primers amplified fungal DNA in the presence of excess DNA from plants 16 days after inoculation (Fig. 3). No fungal DNA was detected in the leaf tissue, which observation was supported by the finding that no fungal growth could be detected from the cut ends of leaf sections of susceptible chickpea cvs 16 days after inoculation with a race 5 isolate of *Foc* (R. M. Jiménez-Díaz unpublished results). Histological examination of the vascular tissue of *Foc*-infected chickpea cvs revealed that there were relatively few fungal structures in stem tissue compared to root tissue after 20 days [21], and this would appear to be reflected in the amounts of PCR products obtained, although quantitative PCR would be required to check this. The wilt-specific primers were highly successful in identifying plant tissue infected with Foc isolate race 5 and the sensitivity of the technique could be increased by hybridization. Four of these amplified samples appeared to have no discernible PCR products, but the presence of the wilt-associated DNA marker was shown by Southern hybridization using the labelled 1.5 kb fragment. In all cases, uninfected plant tissue and plants infected with yellowing-inducing isolates generated no detectable amplification products and no products were discernible by probing.

These results have shown that *Foc* can be detected *in planta* by PCR techniques, and that a specific pathotype can be distinguished. Other studies involving attempts at distinguishing between isolates of *Foc* have shown that there is no consistent difference in the mtDNA of *Foc* [37], and that all isolates analysed so far appear to belong to one vegetative compatibility group [34]. Furthermore, probing of seven different restriction

digests of *Foc* genomic DNA with a heterologous retrotransposon from *C. fulvum* [8, 30] revealed no difference between isolates 8012 (race 5, wilt-inducing) and 7802 (race 0, yellowing-inducing). These results suggest that SCAR-PCR analysis based on RAPD bands, in the absence of extensive gene sequence data, appears to be the most discriminatory form of *in planta* detection for wilt-inducing isolates of *Foc*.

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