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Rapid diagnosis of apple proliferation mycoplasma-like organism using a polymerase chain reaction procedure

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The rapid diagnosis of apple proliferation mycoplasma-like organism, using polymerase chain reaction amplification of part of the 16S rRNA gene is reported. Apple proliferation MLO can be detected in as little as 0·3 g of diseased tissue. Results compare favourably with those obtained by other PCR-based methods. Operational speed, sensitivity, specificity and ease of performance of the method, with particular reference to indexing purposes, are discussed.

INTRODUCTION

Apple proliferation is widespread in Europe. The causative agent is a mycoplasma-like organism (MLO) transmissible from diseased tree tissues to periwinkle (Marwitz *et al.*, 1974; Carraro *et al.*, 1988). The disease is invading apple growing areas.

Because of the economic importance of apple proliferation, particularly in northeastern Italy, rapid and easy diagnostic methods are urgently needed, to identify causative agents, vectors, etc., and to solve practical problems such as checking disease-free material and monitoring the spread of the disease.

Traditionally, MLO diseases have been distinguished as involving virescence and phyllody, or 'decline', the latter group including apple proliferation. The introduction of DNA–DNA hybridization with cloned chromosomal and extrachromosomal DNA as probes has supplied a molecular basis for distinguishing MLOs (Davis *et al.*, 1988; Lee & Davis, 1988; Kuske *et al.*, 1991a, b). DNA probes have also been used for diagnostic purposes. For the specific detection of apple proliferation MLO, Bonnet *et al.* (1990) cloned chromosomal DNA fragments and used the recombinant DNA (labelled with ^{32}P) in dot–blot hybridizations.

In recent years, methods based on restriction fragment length polymorphism (RFLP) analysis of the polymerase chain reaction (PCR) amplified 16S ribosomal RNA gene (16S rDNA) have been proposed. These have allowed the identification of five (Ahrens & Seemüller, 1992) or seven (Schneider *et al.*, 1993) groups of related MLOs.

Because of their sensitivity, PCR methods are also useful in diagnostics. Some workers have sequenced the recombinant plasmids used as DNA probes and have devised PCR primers that are both sensitive and disease-specific. However such assays are only available for some 'virescence'-type MLOs (Deng & Hiruki, 1990; Schaff *et al.*, 1992); at present the diagnosis of other MLO diseases with PCR implies the analysis of an amplification product by restriction endonuclease digestion and acrylamide gel electrophoresis (Ahrens & Seemüller, 1992) or by DNA–DNA hybridization with oligonucleotide probes (Firrao *et al.*, 1993).

In this note we describe a one-step PCR diagnostic assay for the detection of apple proliferation MLO in plant tissues, based on oligonucleotide sequences derived from the variable regions of the 16S rRNA gene.

MATERIALS AND METHODS

Samples

Shoots were collected from a total of 12 apple plants (cvs Summer Red, Golden Delicious, Jonagold and Red Chief) in June 1992, in an orchard in Gemona (Udine), Italy. Plants were labelled as infected or not infected on the basis of symptoms shown in previous years and recorded by Dr L. Carraro of this Department, who also made available a healthy apple, cv. Golden

Delicious, maintained in the greenhouse and used as a control.

Periwinkle plants infected with apple proliferation MLO (Italian isolate), clover phyllody, plum leptonecrosis, tagetes witches' brooms, grapevine yellows and periwinkle virescence were supplied by Dr L. Carraro. Periwinkle plants infected with apple proliferation MLO (German isolate), western X and aster yellows were kindly provided by Dr E. Seemüller (Institut für Pflanzenschutz im Obstbau, Dossenheim, Germany) through Dr L. Carraro. For details on these, see Firrao *et al.* (1993).

DNAs extracted from Asian (nashi) pear with decline symptoms and from peach with rosette symptoms were kindly supplied by Dr C. Poggi Pollini (Istituto di Patologia Vegetale, Bologna, Italy) and Dr C. Marcone (Istituto di Patologia Vegetale, Napoli, Italy), respectively.

DNA extraction

DNA was extracted from 0·3 g of fresh young entire leaves and stems of healthy and diseased plants following the protocol of Doyle & Doyle (1990). Nucleic acids were dissolved in 100 μl TE (10 mM Tris, 1 mM EDTA, pH 8). Twenty μl were run on a 0·7% agarose gel in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8) to check nucleic acid amount and quality.

Group-specific PCR followed by oligonucleotide probing

Two μl of the 100 μl obtained as above were added to a 100 μl PCR mixture containing 10 mM Tris-HCl, pH 8·3, 50 mM KCl, 1·5 mM $MgCl_2$, 10 μg gelatine, 0·1 mM each dNTP (Boehringer Mannheim, Germany), 400 ng each primer P1 and P4 (Firraro *et al.*, 1993) and water to 98·5 μl, overlaid with three drops of mineral oil. PCR reactions were heated to 95°C for 10 min, the temperature was lowered to 80°C and 1·5 μl of 1 U/μl *Taq* polymerase (Boehringer) added to each tube. Amplification was carried out in a PREM III machine (LEP Scientific, Milton Keynes, UK) with 30 cycles of: denaturation (94°C) for 30 s, annealing (42°C) for 30 s and extension (72°C) for 30 s. The final extension step was for 10 min at 72°C. The sequence of the apple proliferation MLO 16S rDNA fragment amplified using primers P1 and P4 is deposited as accession number X72206 in the EMBL–GenBank database.

The same DNA extracts were also amplified using the MLO 16S rDNA specific primer pair (called hereafter A1 and A2) devised by Ahrens & Seemüller (1992) under the conditions described by the authors.

After cycling, 5 μl were added to 95 μl 2 × SSC (1 × SSC is 150 mM sodium chloride, 15 mM sodium citrate) and the resulting solution was vacuum filtered on Nylon membrane (Boehringer) using a Milliblot 96-well dot–blot manifold (Millipore, Bedford, MA, USA). When dry, the membrane was sequentially placed on 3MM paper sheets (Whatman, Maidstone, UK) saturated with 1·5 M NaCl, 0·5 M NaOH (for 5 min), with 0·5 M Tris-HCl pH 7·4 (for 30 s) and with 0·5 M Tris-HCl pH 7·4, 1·5 M NaCl (for 5 min). It was then dried again and baked for 30 min at 120°C. Prehybridization was carried out in 5 × SSC, 0·1% *N*-laurylsarkosine, 0·02% sodium dodecyl sulphate (SDS), 1% blocking reagent (Boehringer) at 42°C. After 1 h the prehybridization solution was discarded and substituted with 1 ml of a solution of the same composition containing about 20 pmol of the oligonucleotide probes. Two 24-nucleotide probes specific for AP, AP1 and AP2 (Firrao *et al.*, 1993) were labelled with digoxigenin using the DIG oligonucleotide 3'-end labelling kit (Boehringer) following the manufacturer's guidelines. Hybridization was allowed to proceed for 4 h at 42°C. Washings were 2 × 5 min in 2 × SSC, 0·1% SDS followed by 2 × 5 min in 0·1 × SSC, 0·1% SDS. The washing temperature was 42°C for AP1 and 46°C for AP2. The digoxigenin-labelled probe on the membrane was detected with a chemiluminescent substrate following the manufacturer's guidelines (Boehringer).

Apple proliferation-diagnostic PCR (AP3–AP5 PCR)

Two μl of the 100 μl obtained as described above from 0·3 g of tissue were added to a 50 μl PCR reaction containing 10 mM Tris-HCl, pH 8·3, 50 mM KCl, 2·5 mM $MgCl_2$, 10 μg gelatine, 300 ng each of primer A3 and AP5, 0·24 mM each dNTP (Bochringer) and water to 48·8 μl, overlaid with three drops of mineral oil. The sequence of the primers was: AP3 5'-CTAAAACTCACGCTT-CAGCTACTC-3' and AP5 5'-TGAGATTTGC-TAAAACTCACGCTT-3'. PCR reactions were heated to 95°C for 10 min, the temperature was lowered to 80°C and 1·2 μl of 1 U/μl *Taq* polymerase (Boehringer) added to each tube. Amplification was carried out with 25 cycles of denaturation (94°C) for 30 s, annealing (63°C)

for 30 s and extension (72°C) for 30 s. The final extension step was 10 min at 72°C. After cycling 6 μl of the PCR product were run in a 1·5% agarose gel in TAE buffer.

RESULTS

The positions of the oligonucleotides used as probes or PCR primers in the apple proliferation MLO 16S rDNA molecule are shown in Fig. 1. Oligonucleotides AP1 and AP3 (AP3 is the reverse-complement of AP1) are located in the variable region called V7 by Gray *et al.* (1984), which is included between positions 589 and 650 in the corresponding sequence of the 16S rRNA of *Escherichia coli*. AP2 and AP5 are located in the variable region called V9 (Gray *et al.*, 1984), which ranges from positions 1239 to 1298 in the *E. coli* 16S rRNA. AP5 is nine nucleotides downstream with regard to AP2 and was synthesized because the AP3–AP2 pair did not produce amplification (not shown). Figure 1 also reports the positions of primers A1–A2 (Ahrens & Seemüller, 1992) and P1–P4 (Firrao *et al.*, 1993).

PCR carried out using the AP3–AP5 pair (AP3–AP5 PCR) gave rise to an amplified fragment of about 670 bp. This amplification was achieved when DNA extracted from apple proliferation-infected periwinkles was added to the PCR reaction (Fig. 2, lanes 2 and 3). No amplification was observed with DNA extracted from healthy plants or those infected with different MLOs (Fig. 2, lanes 4 to 9, 14 and 15), with the exception of the plum leptonecrosis-infected periwinkle (Fig. 2, lane 10) and the nashi pear with decline symptoms (Fig. 2, lane 13). The samples collected from trees that had shown apple proliferation symptoms in the previous years (L. Carraro, unpublished data) reacted positively (Fig. 3, row A, lanes 1 to 8), although symptoms were weak at the moment of sampling. None of the symptomless trees (Fig. 3, row A, lanes 9 to 12) or healthy control trees (Fig. 3, row A, lane 13) gave a positive amplification signal.

The same extracted DNA was also employed for the general amplification of mollicute 16S rDNA with primers P1–P4 and of MLO 16S rDNA with primers A1–A2. The amplification products were then spotted on a nylon membrane and hybridized with the labelled AP-specific probes. The DNA fragments amplified with the P1–P4 pair were allowed to hybridize with both AP1 and AP2 probes, while those amplified with A1–A2 hybridized only with AP2 probes since the shorter DNA fragment did not include region V7. The same samples which gave rise to an amplified DNA fragment in the AP3–AP5 diagnostic PCR were positive in the hybridization assay (Fig. 3, rows B to D).

DISCUSSION

The method described in this paper is based on the amplification of 16S rDNA of apple proliferation MLO. Comparison of the results achieved using AP3–AP5 PCR with other 16S rDNA amplification-based diagnostic assays did not show any significant difference and confirmed the validity of the proposed methodology. However, when using the AP3–AP5 PCR, no further manipulation of the amplified DNA is required and results are immediate and do not require RFLP pattern comparison or hybridization. In particular, the absence of a hybridization step in the assay makes this technique rapid, easy to perform and of automation potential, particularly if coupled to rapid methods for MLO DNA extraction (Firrao & Locci, 1993), while retaining a degree of specificity for the disease agent which was previously achieved only using hybridization (Bonnet *et al.*, 1990; Firrao *et al.*,

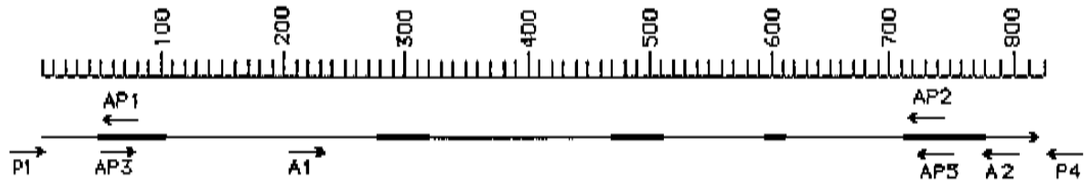

Fig. 1. Schematic representation of the part of the 16S rRNA gene of apple proliferation mycoplasma-like organism amplified by PCR using the primer pair P1–P4 as described in Firrao *et al.* (1993). Thickened lines represent variable regions. The scale gives nucleotide position numbers of the partial 16S rRNA gene sequence (GenBank database accession number X72206). The arrows indicate the hybridization target sites of the oligonucleotides used in this work, whose 3'-ends correspond to the arrow tip.

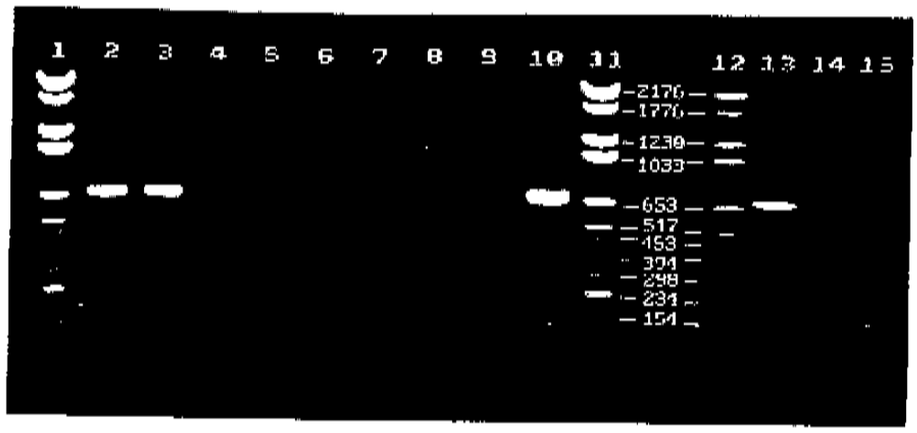

Fig. 2. Agarose gel electrophoresis of amplification products obtained with the primer pair AP3–AP5 from DNA of periwinkles (lanes 2 to 10 and 14) or of natural hosts (lanes 13 and 15) infected with various mycoplasma-like organisms. DNA marker VI (Boehringer) lanes 1, 11 and 12; AP (Italian isolate) lane 2; AP (German isolate) lane 3; healthy periwinkle lane 4; clover phyllody lane 5; tagetes witches' broom lane 6; periwinkle virescence lane 7; western X lane 8; aster yellows lane 9; plum leptonecrosis lane 10; pear (nashi) decline lane 13; grapevine yellows lane 14; peach rosette lane 15.

1993). This assay should therefore be useful for indexing planting material.

Mollicute- or MLO-specific amplification followed by hybridization with disease-specific oligonucleotide probe(s) is suggested as a preliminary general technique in the production of a diagnostic assay for MLOs. Oligonucleotide sequences synthesized on the basis of envisaged behaviour always provided consistent results when used as probes, while they sometimes failed when used as PCR primers, thus requiring the synthesis and testing of additional oligonucleotide(s). In this work the oligonucleotide AP2 worked well as a probe but not as a PCR primer and the oligonucleotide AP5 had therefore to be synthesized to achieve reliable PCR results.

The AP3–AP5 PCR assay was tested for disease specificity using DNAs extracted from plants infected by MLOs representative of the

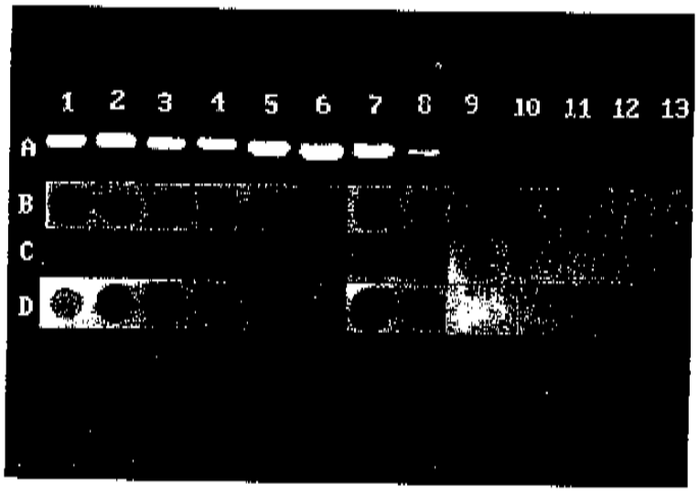

Fig. 3. Agarose gel electrophoresis of amplification products obtained with the primer pair AP3–AP5 (row A) and dot-blot hybridization of amplification products obtained with the primer pair P1–P4 against oligonucleotide probes AP1 (row C) and AP2 (row D) and of amplification products obtained with primer pair A1–A2 against oligonucleotide probe AP2 (row B; lanes 1 to 8, apple proliferation-infected apple trees; lanes 9 to 12, symptom-free apple trees; lane 13, healthy apple tree.

16S rDNA groups I, II, V and VI of the classification proposed by Schneider *et al.* (1993). The reaction was positive with MLOs belonging to group V (AP German strain and AP Italian strain) and with two additional MLOs. The first of these is associated with a disorder similar to the slow 'pear decline' syndrome. No pear decline-associated MLOs were considered in the scheme of Schneider *et al.* (1993), but Davies *et al.* (1993) reported that the pear decline MLO has a 16S rDNA restriction pattern similar to that of AP MLO. The second MLO which was positive, plum leptonecrosis, was classified in group II by Schneider *et al.* (1993). However, the isolate used (PLN-V6) was different from that used in our work and did not hybridize with probes AP1 and AP2 (G.F., unpublished results).

In conclusion, the PCR assay described here is specific for a group of related 'decline' MLOs, including apple proliferation, pear decline and plum leptonecrosis. 'Virescence' MLOs and other 'decline' MLOs (western X disease, grapevine yellows, peach rosette) were not detected.

Part of the 16S rDNA of plum leptonecrosis MLO, sequenced recently, proved to be about 4% different from that of AP MLO (G.F., unpublished results). The continuous increase of MLO 16S rDNA sequence data (Lim & Sears, 1989; Kuske & Kirkpatrick, 1992; Namba *et al.*, 1993; Schneider *et al.*, 1993; G.F., unpublished results) will soon allow precise identification of the sequences that vary among MLOs closely related to apple proliferation. Selection of disease-specific primers will be then possible.

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