Phytopathol. Mediterr. (2004) 43, 228–231

Molecular diagnosis of phytoplasmas

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Summary. Phytoplasmas are wall-less prokaryotes associated with diseases in numerous plant species worldwide. In nature they are transmitted by phloem-sucking insects. Yellowing, decline, witches' broom, leaf curl, floral virescence and phyllody are the most conspicuous symptoms associated with phytoplasmas, although infections are sometimes asymptomatic. Since phytoplasmas cannot be cultured *in vitro*, molecular techniques are needed for their diagnosis and characterization. The titer of phytoplasma cells in the phloem of infected plants may vary according to the season and the plant species, and it is often very low in woody hosts. Different DNA extraction procedures have therefore been tried out to obtain phytoplasma DNA at a concentration and purity high enough for effective diagnosis. DNA/DNA hybridization methods were reported in the nineties to be appropriate for the detection of phytoplasmas, but at present PCR is considered the most suitable. Universal and group-specific primers have been designed on the rRNA operon of the phytoplasma genome and on plasmid sequences. RFLP analysis of the obtained amplicons has classified these pathogens into major 16Sr RNA groups. Group-specific primers have also been designed on other genomic sequences. PCR is a very sensitive technique, but due to the low titre of phytoplasmas a further increase in sensitivity may be required for accurate diagnosis. This is routinely obtained with a second round of PCR (nested PCR). The drawback of nested PCR is that there is a greater chance of obtaining false positives due to contamination. Many authors have therefore developed protocols based on hybridization (PCR/dot blot) or serological approaches (PCR/ELISA) to increase the sensitivity and specificity of the direct PCR, reducing the risks due to nested PCR. Real time PCR protocols may also improve the sensitivity and specificity of the direct PCR assay.

Key words: PCR, real time PCR, hybridization, specific detection.

Introdution

Phytoplasmas are wall-less prokaryotes associated with diseases in numerous plant species worldwide (Lee *et al*., 2000). In nature they are transmitted by phloem-sucking insects. Yellowing, decline, witches' broom, leaf curl, floral virescence and phyllody are the most conspicuous symptoms associated with phytoplasmas, although sometimes infections are asymptomatic. Since phytoplasmas cannot be cultured *in vitro*, molecular techniques must be used for their diagnosis and characterization. The titer of phytoplasma cells in the phloem of infected plants varies according to the season and the plant species, and it is often very low in woody hosts. This is a major obstacle in the diagnosis of these phytopathogens.

Diagnosis of phytoplasmas is routinely done by PCR and can be divided into three phases: total DNA extraction from symptomatic tissue; PCR amplification of phytoplasma-specific DNA; characterization of the amplified DNA by RFLP analysis or nested PCR with group-specific primers.

Total DNA extraction from symptomatic tissue

Different protocols for total DNA extraction have been reported for the detection of phytoplasmas (Ahrens and Seemueller, 1992; Prince *et al*,

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1993; Daire *et al*, 1997; Zhang *et al*, 1998; Green *et al.*, 1999). The main goal of each protocol is to concentrate phytoplasma DNA while reducing enzyme-inhibitory plant polyphenolic and polysaccharide molecules. Most of the protocols involve a phytoplasma enrichment step followed by total DNA extraction, but some authors also suggest to add a proteinase digestion step. The efficacy of some of these protocols in extracting phytoplasma DNA from different plant materials has been tested recently. Palmano (2001) used competitive PCR to compare three protocols in the diagnosis of phytoplasma DNA from herbaceous hosts. She evaluated different parameters (DNA yield, execution time, execution difficulty, need for hazardous reagents) and ranked the protocols tested, showing that a phytoplasma enrichment step though making the protocol more laborious and time-consuming, was necessary to obtain reliable diagnostic results. She suggested that any differences found in the phytoplasma DNA concentration with a given extraction procedure from herbaceous hosts were due to the phytoplasma strain under analysis, to the proper identification of the plant part used for sampling and to variations in phytoplasma titre. At about the same time, Pasquini and co-workers (2001) used nested PCR to evaluate three protocols for total DNA extraction from flavescence dorée (FD) infected grapevines. Following an examination of protocol characteristics (detection efficacy, execution time, number of diagnostic PCRs, need for hazardous reagents, execution difficulty, maximum number of extracted samples per day per operator, cost) they came to the conclusion that a phytoplasma enrichment procedure was needed to consistently improve detection of FD from infected woody hosts. An enrichment step also improved phytoplasma diagnosis in insect vectors, although, possibly due to the high titre of the bacteria in the insect body, diagnostic PCR produced acceptable results even when total DNA was prepared with a quick boiling extraction procedure (Marzachì *et al*., 1998).

Reliable diagnosis of phytoplasmas also depends upon storage conditions of collected samples. Storage of phytoplasma-infected insects at -20°C under acetone instead of ethanol very substantially increased the detection of bacteria (Bosco *et al*., 2002).

PCR amplification of phytoplasma-specific DNA

DNA/DNA hybridization methods were reported in the nineties as lending itself to the detection of phytoplasmas, but PCR is at present the diagnostic technique of choice. Different sequences in the phytoplasma genome have been targeted to design universal and group-specific primers. Until recently (Liefting and Kirkpatrick, 2003) few sequence data were available for the phytoplasma genome, therefore most PCR primers designed for detection were located on a few available genes: the ribosomal operon gene, the elongation factor genes and the ribosomal protein genes. Group-specific phytoplasma primers were also occasionally located on genome portions, most often from the random cloning of a phytoplasma genome for which no obvious function had been predicted (Daire *et al*., 1997; Clair *et al*., 2000; Marzachì *et al*., 2000). Other authors have designed universal primers for diagnosis located on the sequence of plasmids hosted by phytoplasmas (Goodwin *et al*., 1994).

Universal primers based on the ribosomal operon showed varied effectiveness in amplifying the target DNA of phytoplasma-infected plants and insects (Marzachì *et al*., 1998). In 1994 Goodwin and co-workers designed phytoplasma universal primers on the sequence of pPSA45, an aster yellows phytoplasma plasmid known to share sequence homology with extra-chromosomal DNA from other phytoplasmas. These primers consistently amplified a band of the expected size from periwinkles infected with taxonomically different phytoplasmas. They also allowed detection of phytoplasmas in leafhoppers with minimal sample preparation (Goodwin *et al*., 1994; Bosco *et al*., 2002), although no amplification was obtained from FD-infected *Scaphoideus titanus*, and low effectiveness was reported in detecting apple proliferation (AP) phytoplasma in infected psyllids (Bosco *et al*., 2002). The ribosomal operon sequence has also been targeted to design primers for the specific detection of phytoplasmas belonging to defined taxonomic groups (Lee *et al*., 1994, 1995; Marcone *et al*., 1996a, b; Smart *et al*., 1996). These primers are now widely used in nested PCRs following a first amplification round driven by universal ribosomal primers. Since phytoplasmas occur in low ti-

tre in the phloem tissues of their host-plants and their concentration may be subjected to seasonal fluctuations, a nested PCR is often required for diagnostic purposes.

The choice of primer sets for phytoplasma diagnosis by nested PCR mostly depends on the phytoplasma we are looking for. Universal ribosomal primers nested with group-specific primers are extremely useful when the phytoplasma to be diagnosed belongs to a well-defined taxonomic group. Nested PCR with a combination of different universal primers can improve the diagnosis of unknown phytoplasmas present with low titer in the symptomatic host. In this case, the detected phytoplasma is identified by restriction fragment length polymorphism analysis of the amplicon.

Phytoplasma group-specific primers have also been designed on ribosomal protein and elongation factor gene sequences (Gundersen *et al.*, 1996; Schneider *et al.*, 1997; Marcone *et al.*, 2000). These primers, together with 16S RNA sequence analysis, have also been used to group phytoplasmas into a monophyletic clade within the class Mollicutes. Phytoplasma group-specific primers have also been designed on genomic sequences for which no specific function could be predicted. These primers were used to detect phytoplasmas belonging to specific clusters (Daire *et al.*, 1997; Clair *et al.*, 2000; Jarausch *et al.*, 2000a, 2000b; Marzachì *et al.*, 2000).

Variations of the basic PCR protocol concern methods to avoid purification of DNA before PCR, and alternatives to nested PCR assays. Immunocapture PCR protocols have been reported for the diagnosis of AP (Heinrich *et al.*, 2001), while direct hybridization of total phytoplasma-infected insect DNA with a plasmid-derived probe detected group I phytoplasmas with a sensitivity ranging from 53 to 83% depending on the vector species (Bertin *et al.*, 2003). Other alternatives to nested PCR assays have recently been reported, the most important of which are probably PCR-ELISA, PCR-dot blot and real time PCR. PCR-ELISA has been developed for phytoplasmas belonging to different taxonomic groups (Poggi Pollini *et al.*, 1997; Marzachì *et al.*, 2000) and shows good sensitivity with both herbaceous and woody hosts. Different approaches have been followed to develop PCR-dot blot systems. With this assay, PCR amplified products are detected using molecular hybridization with a parental probe. Several combinations of primers/probe have been developed for the diagnosis of phytoplasmas in plants and insects using a plasmid-based system (Goodwin *et al.*, 1994), a non ribosomal stolbur-specific system (Marzachì *et al.*, 2000) and a ribosomal-based system (Bertin *et al.*, 2003). Real time PCR protocols have recently been suggested for the diagnosis of FD and bois noir phytoplasma in field-collected symptomatic grapevines as well as for insect vectors (Marzachì *et al.*, 2003) and for AP-associated phytoplasma (Baric and Dalla Via, 2004).

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Accepted for publication: July 21, 2004