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Discriminating 16Sr groups of phytoplasmas by an oligonucleotide microarray targeting 16S-23S spacer region

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Abstract

A microarray detecting phytoplasma 16Sr groups -I, -II, -III, -V, -VI, -X and -XII was developed based on probes targeting 16S-23S spacer region. Despite that it is considered as more suitable than 16S rDNA for the fine differentiation of phytoplasmas, the microarray did not demonstrate discriminatory potential higher than the only phytoplasma microarray published so far based on 16S rDNA. Nevertheless the method used was cheaper and faster bringing the microarray towards routine analysis.

Key words: phytoplasmas; detection; microarray; 16Sr groups; 16S-23S ribosomal spacer region.

Introduction

For phytoplasma classification the RFLP analysis of 16S rRNA gene is traditionally used, dividing phytoplasmas into ribosomal groups. However, this technique is laborious, sensitive to point-mutations, and only one sample can be analyzed per reaction. Therefore, new techniques have been searched for enabling reliable determination and broader, high-throughput screening of phytoplasmas. Such a technique is represented by microarrays (Hadidi *et al*., 2004), allowing detection of many different sequences in parallel. In contrast to several microarrays discriminating plant viruses (Boonham *et al*., 2008), and bacteria (Huyghe *et al*., 2009), only one microarray detecting phytoplasmas (Nicolaisen and Bertaccini, 2007) was developed so far. Furthermore, not all 16Sr groups reported so far were fully distinguished by this microarray based on 16S rDNA. The authors suggested in accordance with the literature, that probes targeting 16S-23S spacer region may be more appropriate for phytoplasma identification. Therefore, the aim of this study was to develop a microarray to test phytoplasma discriminatory potential of 16S-23S spacer region.

Materials and methods

Phytoplasmas under study were identified strains belonging to 16Sr groups -I, -II, -III, -V, -VI, -X and -XII obtained from collection maintained by A. Bertaccini, Italy. Total DNA was extracted from each sample by NucleoSpin Plant II kit (Macherey-Nagel), diluted 1: 50 in distilled water and 1 µl was used in subsequent PCR. PCR was performed using PCR Master Mix (Fermentas, Vilnius, Lithuania), the 5'-GGGATGGATCACCTCCT TTC-3' was the forward, and the Cy3-5'-ACAAACCC CGAGAACGTATTC-3' was the reverse primer; 35 cycles of 93°C for 30 seconds, 60°C for 30 seconds, 72°C for 1 minute were carried out to achieve the reaction. Amplified labeled fragments (3 µl) were checked by gel-electrophoresis (1% agar; 0.5xTBE) and purified (17 µl) using GenElute PCR CleanUp kit (Sigma-Aldrich) into 70 µl of final volume.

Table 1. Probes designed according to the alignment – position; accession number are listed for orientation only; lower case in probe sequence: random nucleotides.

16Sr group	probe name	probe sequence (5' – 3')	position	accession number
I	1a	AAGAAAGTTTTTCATTGTAACTTGCTTGCAAATTG	1527-1561	HM590616
I	1b	agtccgTATTTGCAACATTTTAATCTTTTTAAactaaag	1601-1626	HM561990
II	2	ttaAAATAATAGTTATTTATCCGGAAACATTAAAGtcc	212-243	DQ471318
III	3a	tcgcaagcgTTCTTTTTTAAGGCATTAAGGaatgcatcc	1558-1578	HQ589207
III	3b	CAGTTTATTTTAAAAGTTATAAGCACTGTCTTAAAAA	1678-1714	HQ221554
III	3c	CCAGTTTATTTTAAAAGTTATAAGCACTGTCTTAAA	1677-1712	HQ221554
V	5	tgtcCAATTTTATATCAGGAAATTATTTACTTCGAAGctga	1712-1744	HQ199312
VI, VII	6/7	cggctaactCCAAAAAAAGGTCCTGCTTAAAcgtacttga	1582-1603	HQ589189
IX	9	agtccgGTTTTTCTGATTTATTTTGTTTTTTtgctaaag	1551-1575	AF515637
X	10	TTTTTTATTTTTTAAGATAAAAATCAATAATGGCTTGGGC	1556-1595	AJ575106
XII, XIII	12a	aacatCCAAAATAGGTCACATCTTAAAAAAGCTCgcattc	1642-1670	HQ589193
XII, XIII	12b	catCCAAATAAAATAGGTCACATCTTAAAAAAAGCTCgca	1668-1701	FJ943262

Two microlitres of DNA targets were sequenced directly (BigDye Terminator kit, Applied Biosystems).

Capture probes (30 - 40 nt) were designed according to the alignment of 16S-23S spacer sequences (GenBank). Shorter probes were extended by random sequences on both ends (table 1). Hybridization of targets (15 µl), microarray washing, scanning and evaluation were done as described previously (Lenz *et al*., 2010). Only spots with signal-to-noise ratio >= 3 were considered as positive.

Results

Twelve capture probes targeting ribosomal spacer of phytoplasma 16Sr groups -I, -II, -III, -V, -VI, -IX, -X and -XII, -XIII were designed. Two of the probes were specific for more than one group (table 1). Sequences of the other groups were not available in GenBank at the time of design. Two double-dots of each probe were printed per microarray. Three replicate samples were hybridized from each of the eight groups tested. All hybridization patterns including cross-hybridization observed (figure 1) correspond to target-to-probe homology revealed by sequencing.

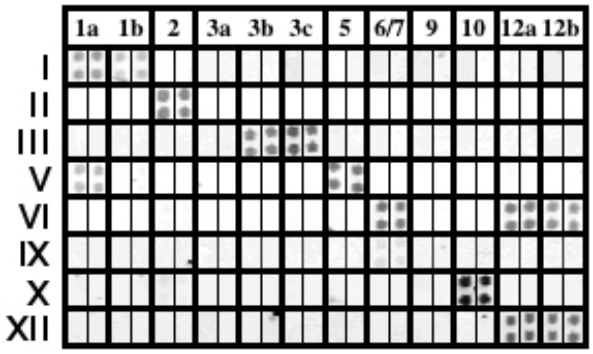

Figure 1. Hybridization results (bleached, inverted colors, contrast adjusted). Rows: 16Sr group of phytoplasma hybridized; columns: name of probes.

Targets of each 16Sr group hybridized to relevant group-specific probes except for probes 3a and 9. For both of these, hybridized groups contained different sequence variant in the probe-targeted site. Furthermore, targets of group 16SrV and 16SrVI hybridized also to the probes designed for groups 16SrI and 16SrXII, respectively. Below-threshold cross-hybridization of 16SrIX group to the probe 6/7 also appeared.

Discussion

In the literature, the usability of 16S-23S ribosomal spacer for better phytoplasma discrimination has been suggested several times. To test this possibility, the microarray targeting different 16Sr groups was developed. Not all 16Sr groups posses unique regions of sufficient length and three of the probes (6/7, 12a, 12b) were not specific for one group only. Furthermore, probe 1a showed significant similarity to 16SrV group in hybridization experiments, and targets of 16SrVI group hybridized to probes 12a and 12b, also. Despite these unexpected cross-hybridizations, all targets hybridized to group-specific probes except of group 16SrIX, variability of which need to be targeted by additional probes.

Compared to the only microarray published so far detecting phytoplasmas based on 16S rDNA (Nicolaisen and Bertaccini, 2007), ribosomal spacer does not provide more discriminating potential. Given that 23S rDNA is much more conserved than both of these, microarray for proper phytoplasma determination has to be based on the other less-conserved genes.

By contrast to the two-step Cy3-dNTPs labelling technique and overnight hybridization used in the previous mentioned paper, the microarray developed here used single PCR Cy3-primer labelling and one-hour-only hybridization, making the process of phytoplasma determination cheaper and faster, thus shifting it more towards a real application.

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