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Short communication

Discrimination of phytoplasmas using an oligonucleotide microarray targeting *rps3*, *rpl22*, and *rps19* genes

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ABSTRACT

Phytoplasmas are economically important pathogens of various fruit trees. Reliable techniques therefore are needed for their detection and discrimination. For this purpose, an oligonucleotide microarray targeting the ribosomal protein genes *rps3*, *rpl22*, and *rps19* of phytoplasmas was tested. Following PCR (35 cycles) of total DNA from phytoplasma-infected plants with Cy5-labelled primer, the microarray reliably detected 16Sr groups I, II, III, V, VI, VII, IX, X, and XII in single infections as well as six different mixed infections (I + II, I + III, I + V, I + VII, I + IX, and I + X) prepared artificially by mixing DNA prior to PCR. It also successfully classified 16Sr groups from field samples collected in the Czech Republic (16Sr groups I, III, and mixed infection I + X). Despite that it did not succeed in distinguishing another two artificial mixed infections (I + VI and I + XII), the microarray developed here provides a suitable alternative to rRNA-based microarrays published previously and where only single infections from experimental hosts were tested. It also advances the technique by targeting single-copy genes with higher inter-group variability than those of 16S rRNA or ribosomal spacer. The usability of the microarray in comparison with DNA barcoding and terminal restriction fragment length polymorphism techniques is discussed.

1. Introduction

There are 28 different groups of phytoplasmas known today based on restriction fragment length polymorphism (RFLP) analysis of the 16S rRNA gene (Wei et al., 2007). RFLP analysis, however, is laborious, sensitive to point mutations, and requires using a region more than 1 kb long. Although other molecular methods – such as reverse transcription PCR (Margaria et al., 2007), real-time PCR (Baric et al., 2006; Crosslin et al., 2006; Saracco et al., 2005), molecular beacons (Firrao et al., 2005), or reverse dot blot analysis (Lessio et al., 2007) – have been employed, these have been able only to detect the presence of one particular group, or of phytoplasmas in general.

Therefore, a method that distinguishes among several different phytoplasmas within a reasonable amount of time is needed for routine screening. One possibility is to use DNA microarrays. However, only two microarrays have been developed for detecting phytoplasmas (Nicolaisen and Bertaccini, 2007; Lenz et al., 2011). Each of these microarrays targets a different part of the ribosomal (rRNA) operon. Due to the low polymorphism of the rRNA operon, it was not possible to distinguish between the 15 groups of phytoplasmas tested in the two earlier studies. Thus, the above authors suggested, that the *tuf*, *secY*, or ribosomal protein (*rp*) genes were more suitable for such purposes. In this study, the *rp* genes, particularly *rps3*, *rpl22*, and *rps19*, were chosen from among the suggested genes due to their high variability among the 16Sr groups. Nevertheless, the sequences of these genes in GenBank had been classified into just 10 groups as at the time of designing the probes (2012), and therefore the microarray developed here targets only 10 groups.

The pros and cons of the microarray are compared to those of the two previously published microarrays. In addition, the microarray's usability is discussed in the context of newly developed DNA barcoding and terminal RFLP.

Abbreviations: SNR, signal-to-noise ratio; T-RFLP, terminal restriction fragment length polymorphism.

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2. Materials and methods

The studied samples were classified strains that were obtained from an *in planta* collection maintained in periwinkle (*Catharanthus roseus*) by A. Bertaccini, Italy (Table 1). Genomic DNA was isolated using the NucleoSpin Plant II isolation kit (Macherey–Nagel, Germany) according to the manufacturer's original protocol. Isolated DNA was quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, USA).

The sequences of the *rps19*, *rpl22*, and *rps3* phytoplasma ribosomal protein genes were retrieved from GenBank and aligned using the POA alignment program (Lee et al., 2002). The probes (35–50 nt) were designed manually within conserved regions in order to capture the most GenBank sequence variants within the targeted group. To fulfil this requirement, each probe had to have both ≥75% homology and ≥15 nt of identity in uninterrupted succession with the targeted group only.

The probes were spotted (BadenBiotec, Germany) onto aldehyde-coated plastic slides (HTA™ Slide12, Greiner Bio-One, Germany). Each probe (~10 pmol) was spotted on the microarray as two separated double-dots.

Primers amplifying the targeted region were designed manually to have ≥75% homology to the majority of phytoplasma sequences and adjusted to have similar Tm using primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Isolated DNA (1 μl) was amplified using PPP-MasterMix (TopBio, Czech Republic) according to the manufacturer's protocol, except that 1 μl of 25 mM $MgCl_2$ was added. For the detection of single infections, the F8a and F8b forward primers with the Cy5-labelled reverse primer R4 (Table 2) were used in a single reaction. Artificially mixed infections and field samples were amplified adding F7 forward primer to the primer cocktail. The PCR conditions were as follows: 95 °C for 3 min; 35 times in the sequence 95 °C for 30 s, 55 °C for 90 s, 72 °C for 2 min; and 72 °C for 10 min. The products were held at 4 °C thereafter. Targets (5 μl) were checked by gel electrophoresis and purified (15 μl) using the GenElute PCR CleanUp Kit (Sigma–Aldrich, USA) for a total final volume 70 μl. Two microliters of PCR product were sequenced directly (BigDye Terminator Kit, v.3.1, Applied Biosystems, USA) with the corresponding primers.

The artificial mixed infections were prepared by combining the same concentrations of selected 16Sr groups. For 16Sr groups I, III, V, and X, corresponding subgroups in the study were mixed together prior to this step and the resulting group-specific mixtures (e.g. I.A + I.B + I.C + I.F) were then used to prepare the artificial mixed infection. One microlitre from each artificial mixture was PCR amplified and purified as described above.

The microarrays were hybridized (1 h), washed, and scanned as described previously (Lenz et al., 2008). SNR values were computed as the background subtracted signal of the particular spot divided by the standard deviation of the background. The signal was considered positive if the SNR was ≥3.

Table 1
Phytoplasma isolates used in this study.

16Sr-subgroup	Phytoplasma	GenBank accession[a]
I-A	Chrysanthemum yellows (Ca. Phytoplasma asteris)	JQ900566
I–B	Dwarf aster yellows (Ca. Phytoplasma asteris)	JQ900567
I–C	Potato purple top (Ca. Phytoplasma asteris)	JQ900568
I–F	Apricot chlorotic leafroll (Ca. Phytoplasma asteris)	JQ900569
II-A	Peanut witches' broom	JQ900570
III-A	Green Valley X disease	JQ900571
III-H	Poinsettia branching factor	JQ900572
V-A	Elm yellows (Ca. Phytoplasma ulmi)	JQ900573
V-E	Rubus stunt	JQ900574
VI-A	Potato witches' broom	JQ900575
VII-A	Ash yellows (Ca. Phytoplasma fraxini)	JQ900576
IX	Picris echioides yellows	JQ900577
X–B	German stone fruit yellows	JQ900578
X–C	Pear decline (Ca. Phytoplasma pyri)	JQ900579
XII	Grapevine yellows	JQ900580
I	field sample from *Brassica napus*	KP064033
I	field sample from *Trifolium pratense*	KP064035
I	field sample from apple tree	KP064032
III-B	field sample from *Echinacea purpurea*[b]	KP064034
X-A + I–C	field sample from apple tree[c]	N.s.

[a] GenBank accessions refer to the targeted sequenced region (*rps19*, *rpl22*, *rps3*). N.s. = not sequenced in this work, 16Sr-subgroups determined by RFLP-analysis.
[b] Franova et al., 2013b.
[c] Franova et al., 2013a.

3. Results

3.1. Specificity of probes and primers

Twenty-seven probes were designed, mainly targeting the *rps3* gene (Table 2) of the phytoplasma 16Sr groups I, II, III, IV, V, VI, VII, IX, X, and XII. No probes were selected for the other 16Sr groups (VIII, XI, and XIII–XXVIII), for which sequences of the targeted genes were not available in GenBank at the time of design (2012). Based on the theoretical hybridization limits (75% homology and 15 nt of identity), each probe was able to capture all GenBank sequence variants within the targeted group (Table 2, column "sensitivity") with the exception of probes for 16Sr groups IX and XII. The latter were designed as probe pairs (XIIa + XIIb and XIIc + XIId), with each probe in the pair targeting a different sequence variant of the same region. All the probes designed were theoretically specific (fulfilling the hybridization limits) for the targeted 16Sr group only.

To cover the variability of the phytoplasma sequences, two versions of the forward primer, F8a and F8b (Table 2), were used with the reverse primer in a single PCR run targeting a region 1230–1390 nt long (length depending on the particular 16Sr group).

3.2. Sensitivity of the microarray

To assess technical limitations of the microarray, tests on the dilution series of Cy3-anti-Ia and Cy3-anti-IIIa2 oligonucleotides (complementary to probes Ia and IIIa2, respectively) were carried out. The minimum number of molecules providing positive signal (SNR ≥ 3) was on the order of 10^6 in both cases (Fig. 1). The average SNR for the remainder of the probes was 0.3 at this dilution.

To obtain positive signal (SNR ≥ 3), the amount of total DNA used for PCR (in 1 μl of sample) ranged from 1.5 ng to 28.0 ng for the experimental host and 9.8 ng–35.3 ng for the field samples. However, no dependence was found between the SNR obtained and amount of total DNA used.

3.3. Detection of single infections

Three replicate samples of each strain hybridized significantly (with SNR ranging from 4.3 to 66.7) to the probes targeting the corresponding 16Sr group (Fig. 2). In particular, all positive signals (SNR ≥ 3) were obtained from the probes sharing ≥95% homology and ≥17 nt of identity with the targets. Similarly, if the theoretical hybridization limits (75% homology and 15 nt of identity in a row) were not satisfied, no hybridization occurred. For example, IXa had 87.5% homology but 10 nt of identity, XIIa had 85.0% homology but 14 nt of identity, and XIId had 89.7% homology but 11 nt of identity.

The only exceptions to these hybridization rules occurred for