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Development of a duplex TaqMan real-time PCR for the general detection of phytoplasmas and 18S rRNA host genes in fruit trees and other plants

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Abstract

The detection of phytoplasmas in fruit trees and other important crops is demanded from nurseries and certification agencies. We have therefore developed an efficient assay for general detection of phytoplasmas. This duplex assay combines the phytoplasma detection with an internal control, detecting host gene 18S rRNA in the same reaction. The internal control enables the confirmation of an efficient DNA extraction and the recognition of eventual inhibition of the PCR. In contrast to a cytochrome oxidase (COX) assay, which did not work in fruit trees, 18S rRNA host gene detection was shown to be usable in a broader range of plants. The sensitivity and robustness of the duplex qPCR was evaluated with different dilutions of samples and compared with traditional nested PCR. The duplex qPCR was at least as sensitive as nested PCR and less susceptible to inhibition due to impurities in DNA extracts. The method was successfully employed for the screening of phytoplasmas in samples of fruit tree, raspberry, and grapevine. Typically, phytoplasma positive samples were tested with Ct values of 22-30, whereas Ct values for the internal control were in the range of 13-23.

Key words: TaqMan, real-time PCR, apple proliferation, pear decline, European stone fruit yellows, *Rubus* stunt, 'bois noir', 'flavescence dorée'.

Introduction

For phytoplasma detection real-time fluorogenic PCR (TaqMan) offers considerable advantage in terms of time and sensitivity compared to traditional or nested PCR. Furthermore, real-time PCR machines equipped with different filters allow the multiplex detection of different DNA targets simultaneously in the same reaction. For routine analysis, an internal control enables the confirmation of efficient DNA extraction and the recognition of inhibition of the PCR. Hence, false negative results can be excluded already in the first round of screening when the amplification of the host gene shows sigmoid curves of fluorescence with typical Ct values for the kind of plant tissue analyzed.

Materials and methods

DNA was extracted from phloem tissue of roots of apple and pear trees, leaves and petioles of raspberry and grapevine with 0.5 g of plant tissue ground 1:10 (w/v) with the HOMEX 6 in extraction bags «Universal» (both from BIOREBA) using the CTAB extraction method according to Angelini *et al*. (2001) and finally resuspended in 0.25 ml of 10 mM Tris pH 7.6.

Duplex real-time fluorogenic PCR was performed with the following primers and probes: for general detection of phytoplasmas, the two forward primers JH-F1 and JH-Fall and the reverse primer JH-R were used at a final concentration of 0.3 µM each, the probe JH-Puni labelled with FAM-MGB-NFQ was used at a final concentration of 0.1 µM (Hodgetts *et al*., 2009). For 18S rDNA detection, the sequences of the following primers (forward: 5'-AGAGGGAGCCTGAGAAACGG-3', reverse: 5'-CAGACTCATAGAGCCCGGTATTG-3') and of the probe (5'–ROX-CCACATCCAAGGAAGGCAGCAGG CG-BHQ2-3') were generated with the software Beacon Designer (Version 7.2, Premier Biosoft International) based on the 18S rRNA gene from *Malus domestica* (Accession No. DQ341382) and used at a final concentration of 0.05 µM each. These primers/probes were run in 20 µl reactions employing the hot start Absolute QPCR Mix (ABgene) and the real-time cycler iQ5 (Biorad) with an initial denaturation and enzyme activation of 12 min at 95°C, followed by 40 cycles with 15 sec denaturation at 95°C and 60 sec annealing/elongation at 60°C. FAM and ROX signals were recorded in real-time during each annealing/elongation step.

Results

The general detection of phytoplasmas by real-time PCR according to Hodgetts *et al*. (2009) was evaluated successfully for detection of phytoplasmas in root, leaf or petiole tissue of different plants. These pathogens were equally well detected in both, simplex or duplex assay formats. In table 1, the evaluation of the duplex assay is presented for roots of healthy and with apple proliferation infected apple trees. The simultaneous detection of the 18S rRNA host gene had no effect on the amplification signal of the specific phytoplasma reaction both in terms of Ct value and sigmoid shape and height of the fluorescence curves (not shown) when compared to the simplex reaction with different dilutions of DNA extracts. Often, however, DNA extracts contained inhibitors retarding or inhibiting the amplification and had to be diluted 10-100 X in order to obtain positive reactions (table 1).

Table 1. Sensitivity comparison of simplex and duplex real-time fluorogenic PCR: DNA extracts from root samples of healthy and infected apple trees were tested in different dilutions with the primers/probe for general phytoplasma detection only (FAM signal; simplex reaction), and as duplex reaction in combination with primers/probe for 18sRNA detection (FAM and ROX signal). Data represent Ct mean values of two repetitions per treatment.

Root sample	Dilution of (DNA extract)	Phytoplasma general simplex real-time PCR	Phytoplasma general + 18S rRNA duplex real-time PCR	
		FAM Ct	FAM Ct	ROX Ct
Healthy	undiluted	>40.00	>40.00	19.97
Healthy	1:100	>40.00	>40.00	20.43
Healthy	1:1000	>40.00	>40.00	23.52
infected/healthy mixed (1:10)	undiluted	25.30	26.06	22.31
infected/healthy mixed (1:10)	1:100	30.03	29.98	21.31
infected/healthy mixed (1:10)	1:1000	32.86	32.77	24.16
infected/healthy mixed (1:10)	1:10000	36.34	35.89	27.55
infected	undiluted	>40.00	>40.00	>40.00
infected	1:100	27.31	27.21	20.00
infected	1:1000	29.19	29.22	22.31
infected	1:10000	33.30	32.76	26.03

In routine analysis, the Ct values ranged from 22 - 30 when detecting *Rubus* stunt in raspberry, 'flavescence dorée' (FD) and 'bois noir' (BN) in grapevine, and apple proliferation (AP), pear decline (PD), and European stone fruit yellows (ESFY) in apple, pear and peach, respectively. Detection limit was between Ct values of 38 - 40. The duplex assay was shown to be sensitive enough for detecting one positive sample in a pool of 10 root samples (table 1).

The duplex TaqMan assay of grapevine phytoplasma FD and BN was shown to be at least as sensitive as nested PCR (Smart *et al*., 1996), but less susceptible to inhibitors. Hence, DNA extracts containing inhibitors could be assayed less diluted in the TaqMan assay, resulting in higher detection sensitivity (data not shown).

Discussion

The duplex real-time fluorogenic assay presented here allows in one reaction the reliable general detection of phytoplasmas simultaneously with the host gene in different plants.

First, the host gene cytochrome oxidase (COX) according to Weller *et al*. (2000) was chosen as the target for the internal control. This worked well for grapevine, potato and raspberry but surprisingly not for fruit trees. Therefore, we have developed the internal control based on the 18S rRNA gene of apple that worked also with grapevine and potato (data not shown) and has therefore the potential as a universal host gene control.

The general detection method of phytoplasmas according to Hodgetts *et al*. (2009), detects phytoplasmas with almost the same sensitivity when compared to specific detection of FD, BN, or *Rubus* stunt (data not shown). This system is therefore reliable for a first screening of diverse plant samples. Positive samples might then be further differentiated or confirmed with specific detection if required.

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