Detection of phloem restricted bacteria responsible for strawberry marginal chlorosis (SMC) by real-time PCR in a single assay

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Abstract

Two uncultured phloem restricted plant pathogens, the *γ3* proteobacterium «Candidatus *Phlomobacter fragariae* » and the stolbur phytoplasma (group 16SrXII-A) are associated with strawberry marginal chlorosis (SMC) in France. As "Ca. *P. fragariae*" and stolbur phytoplasma induce identical symptoms, the only way to identify the pathogen infecting a given diseased plant is to perform conventional PCR assays. Because using two PCR techniques for detecting separately each of the two bacteria is time consuming and because specificity and sensitivity of the detection test needed to be improved, a new approach using triplex real time PCR was developed for the routine detection of "Ca. *P. fragariae* " and stolbur phytoplasma. The real time PCR has the advantage of being faster reduces the risks of producing false positives. Furthermore, real-time PCR techniques provide the possibility of multiplexing by using probes with different compatible fluorescent dyes. Here, we present a new sensitive Taqman® method which permits the simultaneous amplification of three DNA targets in one test: the *map* gene of stolbur phytoplasma, the *spo*T gene of "Ca. *P. fragariae*" and the *cox* gene of strawberry chloroplast taken as an internal control. The specificity and the efficiency of this method were determined.

Keywords: Strawberry Marginal Chlorosis, Triplex taqman® PCR ,Candidatus *Phlomobacter fragariae*, stolbur phytoplasma.

Introduction

During a survey of the French strawberry production carried out from 1996 to 2001, using conventional PCR detection methods, we showed that marginal chlorosis symptoms could be induced by two different pathogens: "Ca. *P. fragariae*" and the stolbur phytoplasma. "Ca. *P. fragariae*" predominated in French strawberry production fields whereas stolbur phytoplasma was prevalent in nurseries (Danet et al., 2003). However, it was later shown that the 16S-rDNA PCR used for the detection of "Ca. *P. fragariae*" also detected the proteobacterium associated to the Syndrome "Basses Richesses" of sugar beet (SBRp) (Gatineau et al., 2002). In addition, SBRp was recently detected in strawberry plants affected by SMC in northern Italy (Terlizzi et al., 2007) and a new phytoplasma "Candidatus *Phytoplasma fragariae*" that belongs to the same taxonomic group as stolbur phytoplasma had been detected in Lithuania on yellowing strawberry plants (Valiunas et al., 2006). As the 16S primers used for the detection of stolbur phytoplasma are conserved in the 16S-rDNA of 'Ca. *Phytoplasma fragariae*', the PCR assay is expected to detect both phytoplasma species. It was therefore decided to develop a new detection test aimed to be more specific for stolbur phytoplasma and "Ca. *Phlomobacter fragariae*'. Triplex real-time PCR was preferred in order to gain sensitivity and perform the simultaneous detection of the two bacteria as well as an endogenous plant analytical control.

Materials and methods

Plant material: Healthy strawberry plants (*Fragaria* x *ananassa* Dutch) were produced by meristem tip culture and *in vitro* propagation and maintained as a control in a greenhouse. Strawberry plants infected with "Ca. *P. fragariae*" or with stolbur phytoplasma were collected in strawberry production fields or nurseries.

Insect DNA extract: The origin of DNA extracts of insects, carrying proteobacteria related to "Ca. *P. fragariae*" and used to evaluate triplex Taqman specificity for "Ca. *P. fragariae*", have been described by Salar et al. (2009). These extracts consist of DNA of *Pentastiridius leporinus* infected by SBRp, DNAs of *Trialeurodes vaporarorium*, *Diaphorina citri*, *Conomelus anceps* and *Mocydia crocea*.

Plant DNA extraction: Petioles of the most symptomatic leaves were detached with a razor blade and washed with water. Total DNA of one gram of petioles was extracted using cethyl-trimethyl-ammonium bromide (CTAB) (Maixner et al., 1995). The final total DNA pellet was resuspended in 100 µl of TE buffer (10 mM Tris, 1 mM EDTA, pH 7.6). DNA from healthy plants was also extracted as negative control.

Design of oligonucleotides and probes: Primers and TaqMan® LNA probes used for triplex real-time PCR are detailed in Table 1. They were designed using the "beacon designer" software version 5.1. The primers and probe for stolbur phytoplasma were described in a previous work (Pelletier et al., 2009), except that the probe has been modified by LNA (Locked Nucleic Acid) bases (Braasch and Corey, 2001) and extended by 4 bases at the 5' end. The probe was 5' labelled with VIC™ reporter dye. The amplified fragment was 72bp long. Primers and probe for "Ca. *P. fragariae*" were determined by comparing the *spo*T gene sequences of 5 reference bacteria, the SBRp, the *Trialeurodes vaporarorium* proteobacterium, the *Diaphorina citri* proteobacterium, the *Conomelus anceps* proteobacterium, and the *Mocydia crocea* proteobacterium (respectiveGenbank accession number FM992680, FM992677, FM992682, FM992678, FM9926679). The probe was 5' labelled with FAM™ reporter dye. The amplified fragment was 75 bp long. Primers and probe sequences for the amplification of the endogenous control (EC) were determined on the basis of the alignment of *cox* genes of 10 plant species : *Solanum tuberosum, S. lycopersicum, Pisum sativum, Brassica juncea, Oenothera berteroana, Populus tremuloides, Beta vulgaris, Oryza vulgaris* and *Zea mays* (respective Genbank accession numbers X83206, X54738, AF338446, X14409, AY300014, X05465, U77623, DQ381450, X15990 and AF542203). The probe was 5' labelled with TEXAS RED ™ reporter dye. The amplified fragment was 81 bp long. All probes were 3' labelled with a non-fluorescent quencher (BHQ).

Tab. 1 Primer and probe sequences for simultaneous detection of "Ca. *P. fragariae*", stolbur phytoplama and a plant endogenous control by real-time PCR.

Name	Target	Sequence 5'→ 3'
Coxfrag-F	Plant	CGTCGCATTCCAGATTATTCC
Coxfrag-R	mitochondrial	CCCAACTACGGATATATAAGAGC
Coxfrag-TexasRed-LNA	*cox* gene	TEXAS RED-AAAL*TGCZ*AAGGGCATTCCA-BHQ-2
MapStol-F	Stolbur phytoplasma	ATTTGATGAAACACGCTGGATTAA
MapStol-R	map gene	TCCCTGGAACAATAAAAGTYGCA
MapStol-VIC-LNA		VIC-CAE*AE*AE*CL*CE*CE*AAE*TGC-BHQ-1
SpoTphlomo-F	"Ca. *Phlomobacter*	AGGTCATGCCTGTGTTGGAG
SpoTphlomo-R	*fragariae*"	TGGTTTGCCGGTACTTAAC
SpoTphlomo-FAM-LNA	spoT gene	FAM-TGGGACAP*AGGAE*AGGGTTGACGA-BHQ-1

*LNA modified bases:L=C-LNA; Z=T-LNA;E=A-LNA and P=G-LNA.

PCR assays: The current official detection method for "Ca. *P. fragariae*" using the PCR primers pair Fra4-Fra5 was performed as previously described (Zreik et al., 1998). Primers Stol11F3 andStol11R2 were used to perform the current official PCR detection method for stolbur phytoplasma which consists of a direct PCR developed from a protocol previously published for the detection of stolbur phytoplasma in grapevine (Clair et al., 2003). The TaqMan triplex real-time PCR reaction was performed in a final volume of 25 µl comprising 12.5 µl of QuantiTect Multiplex PCR buffer (Qiagen), primers and probes at a final concentration of 0.2 µM and 5 µl of purified DNA. Amplification and detection were performed using the CFX 96 Real-Time system apparatus (Bio-Rad). The thermal cycle consisted in a pre-step of 15 min at 95 °C for Hot Start Taq DNA polymerase activation, followed by 40 cycles of 60 s denaturation at 94 °C and 90 s hybridization and elongation at 59 °C. Each reaction included at least one blank assay without template and two negative controls corresponding to healthy plants. The software Bio-Rad CFX manager was used for fluorescence acquisition and estimation of threshold cycles (Ct). For this estimation, the baseline was automatically set and the fluorescent threshold was set manually for each individual target to intersect with the linear part of all amplification curves.

Evaluation of the Taqman real-time assays efficiencies: The efficiency and the linear range of each real-time PCR reaction were evaluated by constructing dilution curves of DNA extracts from two different samples: one strawberry plant infected with stolbur phytoplasma and one "Ca. *P. fragariae*" -infected strawberry plant. 10-fold serial dilutions were performed and each dilution was tested in duplicates. The slope (k) of the linear regression line between logarithmic values of the dilution factor (x-axis) and estimated Ct values (y-axis) was used to calculate the amplification efficiency, $E = (10^{-1/k}-1) \times 100$ (Pfaffl, 2004).

Results

Specificity of the triplex real-time PCR assay: The specificity of the stolbur phytoplasma real-time PCR assay was previously tested (Pelletier et al., 2009). The specificity of "Ca. *P. fragariae*" real-time PCR was tested on DNA extracts from healthy plant and on DNA extracts from insects carrying proteobacteria phylogenetically related to "Ca. *P. fragariae*". In this case, the Ct value was 27.8 for the strawberry DNA infected by "Ca. *P. fragariae*" whereas no significant signal was observed for the other bacteria (Figure 1).
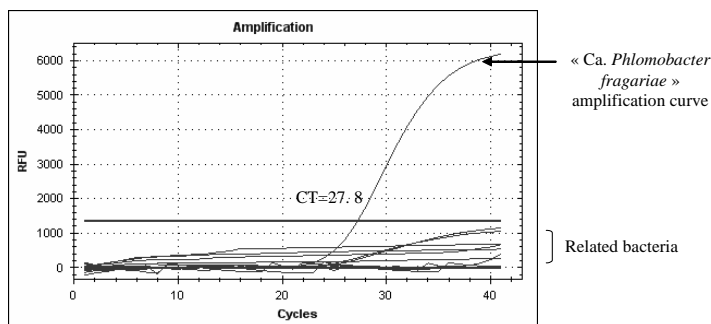
Fig. 1 Taqman® PCR amplification curves of proteobacteria phylogenetically related to "Ca. *P. fragariae*":
SBR proteobacterium, *Trialeurodes vaporarorium* proteobacterium, *Diaphorina citri* proteobacterium,
Conomelus anceps proteobacterium, *Mocydia crocea* proteobacterium

Performance of the triplex real-time PCR assay in comparison with the simplex real-time PCR assay: Calibration lines
were constructed for each target by analyzing ten times serial dilutions of total DNA extracted from one strawberry
plant infected by "Ca. *P. fragariae*" and one strawberry plant infected by stolbur phytoplasma. PCR efficiency values
were 100.6 %, 100.2 % and 101.7 % for the "Ca. *P. fragariae*", stolbur phytoplasma and EC target respectively when
they were tested in simplex real-time PCR and 101.71 %, 102.5 % and 112.9 % when they were tested in a triplex real-
time PCR. Therefore, these results indicated that multiplexing did not reduce the efficiency value.

Triplex real-time PCR detection sensitivity in comparison with the conventional PCR official test: Sensitivities of the
triplex real-time PCR assay and the official PCR test were compared by analysing the same serial dilutions. Figure 2
shows results obtained for "Ca. *Phlomobacter fragariae*" and stolbur phytoplasma detection on these dilutions. For "Ca.
Phlomobacter fragariae", the triplex Taqman PCR gave positive amplification up to a dilution of 10^{-4} whereas the
official PCR detected the bacterium DNA up to a dilution of 10^{-2}. For stolbur phytoplasma, the Triplex Taqman PCR
gave a positive amplification up to a dilution of 10^{-6} whereas the official PCR detected the phytoplasma DNA up to a
dilution of 10^{-5}. The sensitivity of the triplex real-time PCR is therefore 100 times higher than the current PCR for the
"Ca. *Phlomobacter fragariae*" and 10 times higher for the detection of the stolbur phytoplasma.
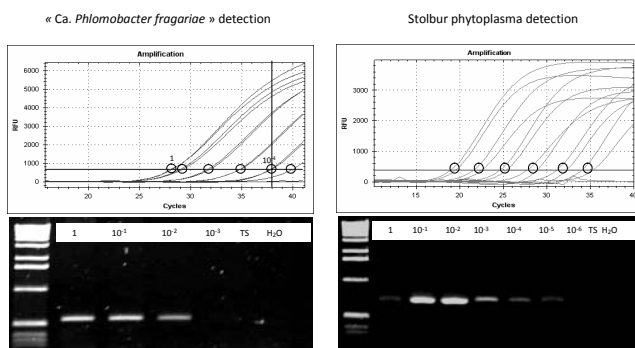

Fig. 2 Taqman® PCR amplification curves of tenfold dilutions of strawberry plant DNA infected with
"Ca. *P. fragariae*" or with stolbur phytoplasma (top). Agarose gel electrophoresis of the official
conventional PCR assay on the same DNA dilutions (bottom)

Discussion

A new real-time PCR method was developed for the simultaneous detection of "Ca. *P. fragariae*" and stolbur
phytoplasma in strawberry plants with an endogenous control. The use of the TaqMan technology allowed the
multiplexing of three different targets: *spo*T gene of "Ca. *P. fragariae*", *map* gene of stolbur phytoplasma and *cox* gene

for the endogenous control. Specificity was promoted by the use of LNA conjugates (Braasch and Corey, 2001) and by the choice of a non-ribosomal target. Indeed, variability in the *spo*T gene helped discriminate "Ca. *P. fragariae*" from among bacteria of its phylogenetic group, whereas the PCR detection based on the 16SrDNA did not (Foissac et al., 2000). The triplex real-time PCR showed lowered limits of detection in comparison to the official PCR assay: up to 10 and 100 times lower for the stolbur phytoplasma and "Ca. *P. fragariae*" respectively. This new multiplex assay, improved both in specificity and sensitivity, will be used to re-evaluate the relative incidence of the two phloem-limited bacteria associated with SMC in French strawberry production.

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