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Universal primers for plasmid detection and method for their relative quantification in phytoplasma-infected plants

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Abstract

Rep gene occurring in the majority of plasmids associated with phytoplasmas was selected as a target for real-time PCR together with single-copy *Tuf* gene from the phytoplasma genome. Rep-specific primers amplified products from host plants infected with phytoplasmas of groups 16SrI, -II, -III, -V, -X, and -XII. Relative concentrations of plasmids ranged depending on strain from 1-2 copies to several hundred copies per each phytoplasma studied. Primers targeting the *rep* gene are proposed for direct PCR detection of phytoplasma plasmids from experimental periwinkle host as well as from natural plant hosts.

Key words: plasmid, universal primers, *rep* gene, phytoplasma.

Introduction

In addition to the relative small genome of 530 to 1,350 kbp (Marcone *et al*., 1999), circular extrachromosomal DNAs of various sizes have been detected on phytoplasmas '*Candidatus* Phytoplasma asteris', '*Ca*. P. trifolii', '*Ca*. P. pruni' and '*Ca*. P. australiense'. The origin of these molecules has not been clearly elucidated, but similar A+T total content to that of the host phytoplasma implies a putative phytoplasma origin. The repA or dnaG proteins accompanied by a single-strand DNA-binding protein (ssb) involved in self-replication have been identified by sequence comparison in all of these plasmids.

Observed sequence variability and the occurrence or absence of distinct open reading frames (ORFs) in various beet leafhopper-transmitted virescence phytoplasma strains suggest that the genes carried are not essential to phytoplasma viability and replication, but they may encode other factors that are required at various levels or during different phases of phytoplasms life cycle (Liefting *et al*., 2004).

It has been documented that several plasmids are present in distinct phytoplasma strains: four plasmids from 1.7 to 5.2 (or 7.4) kbp in size have been detected in Western aster yellows phytoplasma and tulelake aster yellows phytoplasma, respectively, in China aster, celery, or periwinkle hosts (Kuske and Kirkpatrick, 1990). Another set of four plasmids 3.8 to 5.1 kbp in size was identified in lettuce infected with aster yellows witches' broom phytoplasma (Bai *et al*., 2006). Different strains of onion yellows phytoplasma contained two or three small plasmids in garland chrysanthemum (Nishigawa *et al*., 2003). No relationship has been observed between number of plasmids and distinct host and/or phytoplasma species or plasmid influence on severity of disease symptoms, but, in fact, no detailed evaluation has yet been performed.

Here we present a PCR amplification method for plasmid screening in phytoplasma infected plants and evaluation of their relative quantification.

Materials and methods

Periwinkle plants infected with phytoplasmas from ribosomal groups 16SrI, -II, -III, -V, -X, -XII as well as collected samples of apple, pear, red clover, red currant, peach, poplar, oak, grapevine, willow, and many herbaceous plants were tested. Total DNA was isolated from 0.1 g of leaf tissue with the NucleoSpin PlantII kit (Macherey-Nagel, Germany) according to the manufacturer's instructions and eluted with 50 µl of water. The single copy *tuf* gene from the phytoplasma genome was amplified as a reference gene with primers fTufu/rTufu (Schneider *et al*., 1997) in parallel with amplification of the *rep* gene localized on plasmid with Rep200/Rep750 primers (table 1). The 20 µl reactions were performed on an iCycler (Bio-Rad) in the presence of 0.3 µM of each primer, 2.5 mM $MgCl_2$, 200 µM of each dNTP, 1X PCR buffer, 1.25 U of DreamTaq DNA polymerase (Fermentas), 1x SYBR Green I, and 1µl of DNA. Cycling conditions were as follows: 95°C for 2 minutes, 40 cycles at 94°C for 30 seconds, 50°C for 30 seconds, and 72°C for 1 minute. The amplification products were electrophoresed on agarose gel (figure 1), excised, extracted with NucleoSpin Extract II kit (Macherey-Nagel, Germany), and sequenced with rep 750 primer using a BigDye sequencing kit (Applied Biosystems).

Results

Amplification products of about 550 bp size were obtained using the Rep220/Rep750 primers in all infected periwinkle plants and also in samples from original host plants. Healthy periwinkle did not produce such amplicon. Nucleotide sequence comparison revealed onion yellows phytoplasma as the most similar sequence.

The threshold value (C_T) for the reference Tuf gene product was around 23, the C_T of Rep ranged from 14 to 23, depending on the sample (figure 2). The calculated plasmid copy number was from 1 to about 500 per single phytoplasma genome.

Table 1. Primers used in this work.

Primer name	5´- 3´sequence	Reference
Rep200	TATATTTAAGATTTAATTATGC	this work
Rep750	ACGTAGGTCATCTAAAATAATAC	this work
fTufu	CCTGAAGAAAGAGAACGTGG	Schneider *et al*., 1997
rTufu	CGGAAATAGAATTGAGGACG	Schneider *et al*., 1997

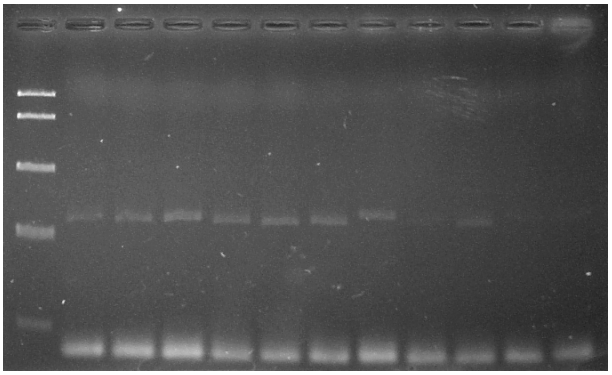

Figure 1. PCR amplification product of grapevine yellows samples with Rep200/Rep750 primers.

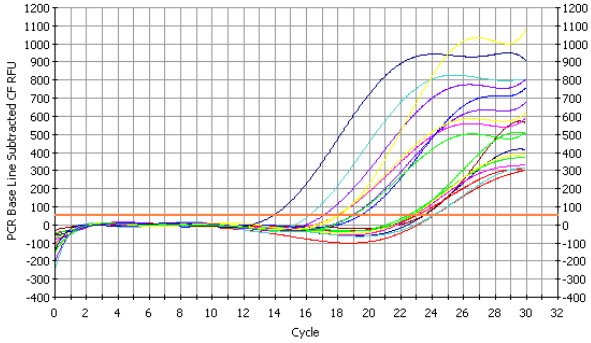

Figure 2. Real-time Amp/Cycle graph of 9 phytoplasma infected samples with Rep and Tuf primers.
(In colour at www.bulletinofinsectology.org)

Discussion

Two different *rep* genes could drive the replication of phytoplasma plasmids. The Rep200/Rep750 primers amplify efficiently the plasmid related to pOYNIM (Nishigawa *et al*., 2003), but not the one related to pBLTVA or to pPaWBNy (Liefting *et al*., 2004; Lin *et al*., 2009). However, both types of plasmid were detected also in one host (Bai *et al*., 2006). Assuming that presence of phytoplasma plasmids is inseparable from phytoplasma itself, the Rep200/Rep750 primers are recommended for routine screening of phytoplasmas in natural hosts.

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