

A New Method to Develop Internal Controls for the Determination of Phytoplasma Concentration

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This study aimed to develop a quick and simple method that generates internal controls from any polymerase chain reaction (PCR) amplified phytoplasma DNA fragment. Non-specific PCR conditions were used to generate unspecific DNA bands with the same primers as the target phytoplasma DNA fragment, but different in their sizes. The method is universal enough to be adaptable for any selected primer pairs. The procedure does not require preliminary knowledge of the sequence or restriction sites of the amplified DNA fragment. Developed internal controls are ligated into a bacterial vector, which can serve as a competitor, to co-amplify with the target phytoplasma DNA in a competitive PCR reaction. Serial dilutions of the internal controls with adjusted concentration and fixed amounts of target templates from phytoplasma-infected plants were amplified together with the same primers to estimate the relative amount of phytoplasma DNA.

Keywords: Phytoplasma, internal control, quantitative PCR, competitive PCR, non-specific PCR conditions.

Phytoplasmas (formerly called mycoplasmalike organisms or MLOs) are unculturable, wall-less bacteria of the class Mollicutes that are causing diseases of several hundred plant species (McCoy et al., 1989; Seemüller et al., 1998) including many perennial fruit crops (Lederer and Seemüller, 1992; Ahrens et al., 1993; Daire et al., 1993).

Only limited information is available on the concentration range of phytoplasma in plant tissue, however phytoplasma concentration can be an important factor from many points of view. For example concentration can be related to pathogenicity, the process of infection or the seasonal pattern of colonisation. Phytoplasma concentration can vary greatly in infected plants. It is often low in woody hosts, many times below the detection level of microscopical (Caudwell and Kuszala, 1992) or PCR detection methods (Marccone et al., 1996) where only nested PCR will give a positive result. Phytoplasma concentrations of apple and poplars have been investigated by DAPI fluorescence microscopy (Kartte and Seemüller, 1991; Berges et al., 1997).

Fruit tree phytoplasmoses are caused by specific pathogens; apple is affected by the apple proliferation (AP) agent, pear is affected by the pear decline (PD) agent and the different stone fruit species are affected by one pathogen, the so-called European stone fruit yellows (ESFY) phytoplasma. All these phytoplasmas belong to the same phyloge-

netic clade, the AP phytoplasma group (Seemüller et al., 1998). Our special attention here is directed to the phytoplasma diseases of temperate fruit crops, since in most fruit growing areas of Europe stone fruit and pome fruits species are severely affected by phytoplasma diseases causing great losses in the value of the crops.

The most suitable quantification method for a non-cultivable organism like phytoplasma is competitive PCR. In this method, the pathogen DNA is amplified together with a so-called internal control, which is distinguishable from the pathogen DNA fragment on the basis of differences in length or restriction sites of the amplification products. Target DNA and the internal standard DNA compete for the same primers during the PCR reaction, so comparison can be made on the basis of relative yield of the two amplification products. Quantification is possible because concentration of the internal control (which is usually a cloned bacterial DNA fragment) is known. Amounts of phytoplasma have been quantified by competitive PCR in infected periwinkles (Pressacco and Firrao, 2000), in aster yellows infected leafhopper (Liu et al., 1994). Berges et al. (2000) studied the concentration of phytoplasma in various plant hosts with the calibration of the internal standard using a culture of *Acholeplasma laidlawii*. The large difference in concentration values measured in their study draws attention to the importance of concentration measurements. Internal control was generated by digesting and internally deleting of a highly conserved 16S rDNA phytoplasma fragment of aster yellows phytoplasma. Preliminary sequence information of a DNA fragment is necessary for the restriction endonuclease based development method of internal controls; moreover this method is limited by the number of restriction sites found on a given fragment. The aim of this study was to develop a method that generates internal controls for any PCR amplified phytoplasma DNA fragment. This universal method would not require knowledge of sequence or of restriction sites, thus allowing for a quick and simple development of phytoplasma internal controls.

Materials and Methods

Sources of plant material and DNA extraction

Samples originated from ESFY and AP phytoplasma reference strains obtained from E. Seemüller, Dossenheim, Germany that have been maintained in the host *Catharanthus roseus* (periwinkle) by periodical graft-transmission. Periwinkle that was maintained in an insect-proof greenhouse was included as a healthy control. DNA from the ESFY reference strain was obtained from young shoots including leaves of diseased *Catharanthus roseus* using the phytoplasma enrichment procedure described by Ahrens and Seemüller (1992). Samples from healthy periwinkles were extracted similarly.

Primers used in PCR and PCR conditions

Three primer pairs were included in our experiments; the universal primer pair fU5/rU3 (Lorenz et al., 1995) that amplify a 800 bp fragment of the 16S rRNA gene, the European stone fruit phytoplasma specific primer pair ECA1/ECA2 (Jarausch et al.,

1998) that amplify a 237 bp nonribosomal fragment, and the Apple proliferation phytoplasma specific primer pair (designed by M. Fodor) Tvf [5'-TCTATTCGTGAAGGTG-GACG-3'] / Tur [5'-TTTGCCTTAGCATTGTCAGC] that amplify a 782 bp fragment of the AP *tuf* gene (Berg and Seemüller, 1999).

All PCR reactions were performed in a PTC-200™ thermal cycler (MJ Research), started with a 1-min denaturation step at 94 °C, and ended with an elongation step for 7 min at 72 °C. Cycle conditions for the generation of non-specific products were as follows in "Slope PCR"; 45 cycles of 30 s at 94 °C, 45 s at 30 °C and rising with 0.6 °C in each cycle, and 60 s at 72 °C. Cycle conditions of competitive PCR and for the amplification of cloned internal control fragments in pGEM vector were as follows; 35 cycles of 40 s at 94 °C, 45 s at 55 °C, and 60 s at 72 °C. Reaction mixtures of 20 µL contained 2.5 µL DNA solution, 0.2 µM of each primer, 100 µM dNTP, 1.5 mM MgCl₂ and 0.5 unit Taq polymerase (Taq DNA polymerase, Promega or Dupla-Taq, Zenonbio) in the reaction buffer supplied by the manufacturer. Reaction mixtures for the development of non-specific fragments have the same composition, with the exception that it contained 5 or 6 µM of each primer, 3 mM MgCl₂, or both in combination. Composition of the reaction mixture in competitive PCR was the same as described above, with the exception that it contained 2 µL plasmid DNA of different dilutions and 2 µL DNA solution extracted from phytoplasma infected plants. PCR amplification products were analysed by electrophoresis on 1% agarose gels. DNA was stained with ethidium bromide and visualised on an UV transilluminator.

Ligation of amplification products into T/A cloning vector, plasmid purification

After agarose gel electrophoresis, fragments were excised from the gel, extracted using the Gel extraction kit (Viogene) and ligated into the pGEM T/A cloning vector according to the protocol of the supplier (pGEM-T Vector Systems, Promega). Recombinant plasmids containing phytoplasma DNA fragments were selected and purified for PCR reaction as described by Sambrook et al. (1989).

Results and Discussion

The main goal of this study was to have DNA fragments suitable for the quantification of phytoplasma in competitive PCR reactions. The ideal method of production of internal controls should be applicable for any primer pair, therefore three very different primer sets were selected; the universal fU5/rU3 primer pair for the amplification of a phytoplasma ribosomal DNA fragment, the AP specific Tvf/Tur primer pair that amplifies the part of the sequence of the *tuf* gene, and the ESFY specific ECA1/ECA2 primer pair designed to multiply a short nonribosomal fragment. Non-specific PCR conditions were used to generate several DNA fragments that could be amplified with the same primers as the target phytoplasma DNA fragment, but different in their size (generally smaller amplification products have been selected). Non-specific PCR conditions were created with different methods: (1) Extremely low annealing temperature (30 °C) for the first cycle, with a gradual increase in the temperature of 0.6 °C/each new cycle ("Slope PCR").

The annealing temperature increased to 57 °C at the last cycle, assuring the specificity of the reaction; (2) 2.5 and 3 fold increase in primer concentration when compared to the 0.2 μ M primer concentration used in standard PCR reactions; (3) Use of a higher MgCl_2 concentration to decrease selectivity: 3 mM MgCl_2 instead of 1.5 mM MgCl_2 proved to be effective in creating non-specific conditions. These methods have been applied alone or in combination (Fig. 1).

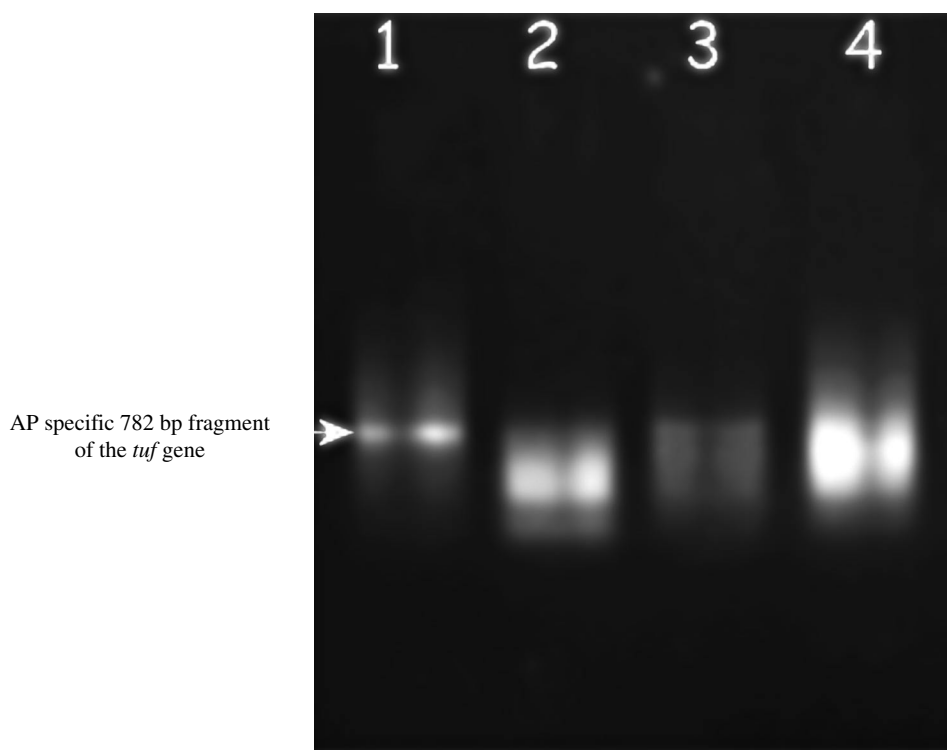


Fig. 1. Electrophoresis of PCR fragments of AP infected periwinkle obtained with primer pair Tvf/Tur using non-specific PCR conditions. Amplification pattern obtained with only "Slope PCR" (1), in combination with increased primer concentration (2) or with increased MgCl_2 concentration (3) or both (4)

A smear could be observed as a result of the amplification of several non-specific fragments. DNA fragments of the excised smear regions of different lines of agarose gel were randomly ligated into a suitable selection vector system. A bulking method helped simplify the selection procedure; DNA of 20–50 isolated colonies were bulked into one sample and subjected to PCR reaction with the primers that were used to generate non-specific fragments. Samples that gave scorable bands were selected and divided into two parts, until 2–3 colonies composed one sample, and finally separate colonies were ana-

lysed by PCR reaction. Recombinant vectors containing one phytoplasma fragment differing in its size from the original DNA sequence were chosen for further application in competitive PCR. An amplification reaction with the original primer pair at the appropriate annealing temperature (56 °C for all the three primer sets) was performed as a final control step (Figs 2, 3).

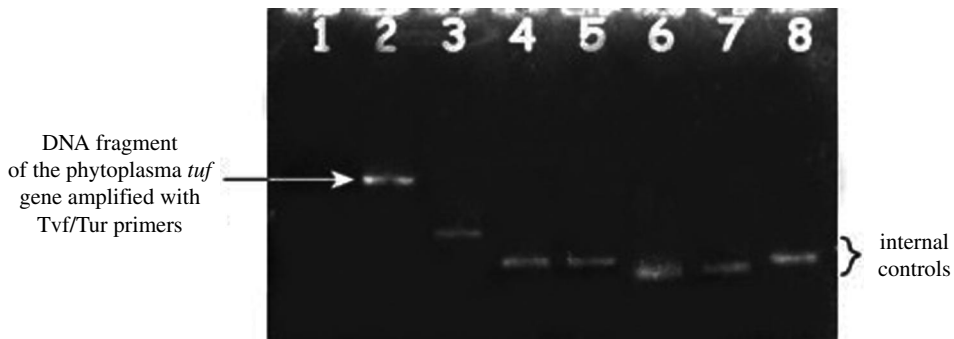


Fig. 2. AP internal controls created using Tvf/Tur primers. Agarose gel electrophoresis of standard PCR reaction of different recombinant pGEM plasmids (lanes 3–8), AP infected *Catharanthus roseus* as a positive control (lane 2) and healthy periwinkle as a negative control (lane 1)

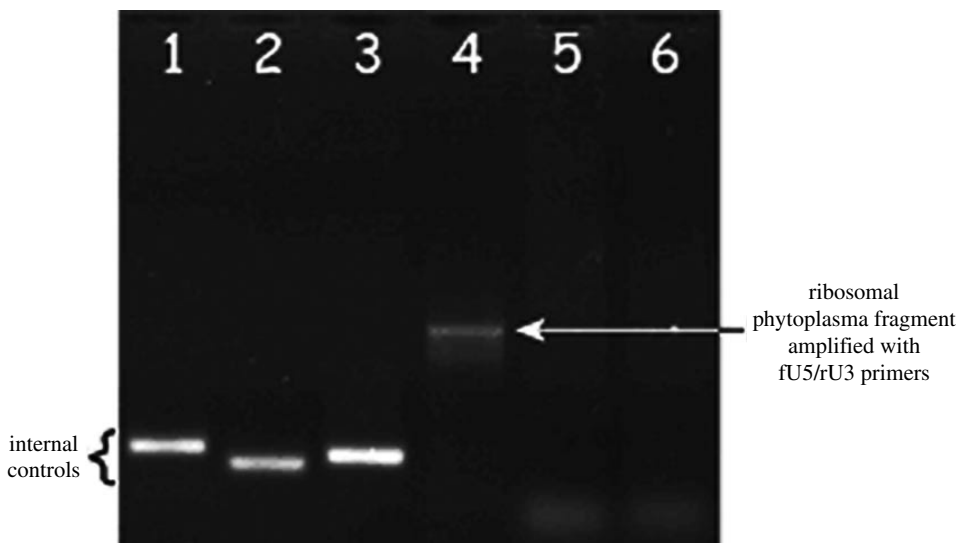


Fig. 3. Universal internal controls created using fU5/rU3 primers. Agarose gel electrophoresis of standard PCR reaction of different recombinant pGEM plasmids (lanes 1–3), ESFY infected *Catharanthus roseus* as a positive control (lane 4) and healthy periwinkles as negative control (lanes 5, 6)

A randomly chosen cloned universal internal control was used as a competitive template for the quantification of ESFY in periwinkle. *Figure 4* shows the results of competitive reactions with a serial (5-fold) dilution of the recombinant plasmid and a fixed amount (2 μ l) of DNA extracted from an infected periwinkle. The two amplification products appear to be of similar intensity in lane 3 corresponding to 5 ng of the competitive template. Since the genome size of the ESFY phytoplasma (\approx 600 Kbp) is about 160 times the size of the recombinant plasmid vector (3800 bp), we estimated the amount of the phytoplasma DNA present in our reaction (2 μ l; from the extraction) approximately at 800 ng.

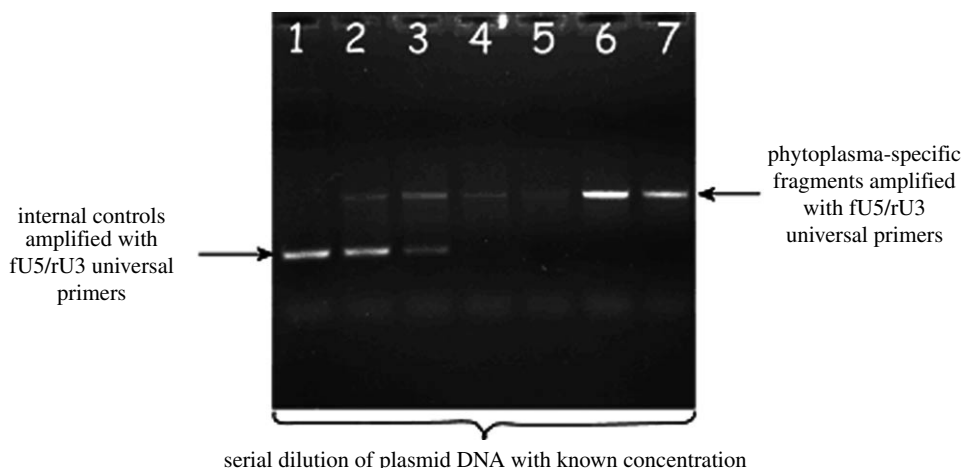


Fig. 4. Agarose gel electrophoresis of products obtained with primer pair fU5/rU3 in competitive PCR. Lanes 1 to 7 show the products obtained with target phytoplasma DNA (upper band) and the dilution of the competitor DNA template (lower band)

This simple and quick method describes the development of phytoplasma internal controls for use in competitive PCR. It is universal enough to be adaptable for any selected primer pairs. The procedure does not require preliminary knowledge of the sequence of the amplified product. Lack of suitable restriction sites of a DNA fragment is not a limiting factor to generate internal controls. Developed internal controls are ligated into a bacterial vector, which is easy to handle, and can serve as a competitor with adjusted concentration, to co-amplify with the target phytoplasma DNA in the competitive PCR reaction. Relative amounts of target DNA can be estimated by comparison with the universal, AP group- and ESFY-specific internal controls developed in this study as competitors.

Acknowledgements

This research was partially supported by OTKA T 025014 and OTKA F 030260 research funds. The authors thank Dr. E. Seemüller for providing ESFY and AP reference strains in periwinkle.

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