Establishment of a Quantitative Real-Time PCR Assay for the Quantification of Apple Proliferation Phytoplasmas in Plants and Insects

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Abstract

A quantitative PCR (qPCR) assay was established for a sensitive and specific quantification of apple proliferation (AP) phytoplasmas in plants and in insect vectors. Different AP phytoplasma-specific primer pairs previously selected in a non-ribosomal DNA fragment of AP phytoplasma were tested. Among these, primer pair AP3/AP4 has been chosen for the qPCR assay because it amplifies a small sized 162 bp fragment of AP phytoplasma and produces no artefact bands. Thus, with these primers the SYBR™ Green technology could be used to monitor the amplification of the PCR products in real-time. The absolute quantification of the phytoplasmas in the samples was done by using the standard curve quantification method. The plasmid pUCI196 containing the chromosomal fragment of AP phytoplasmas from which the specific primers were derived was used as standard. Serial dilutions of the plasmid were done in total DNA extracts of healthy plants and healthy psyllids, respectively. For insects, total DNA of single individuals was extracted and subjected to PCR. Thus, AP phytoplasmas could be quantified in single individuals. For plant material, quantification of AP phytoplasmas was done with reference to a defined fresh weight of the material prior to DNA extraction. The inter-assay and intra-assay reproducibility of the method was analysed by comparing the Ct-values for given samples. The reproducibility was high both with plant and insect samples. Great differences in phytoplasma load could be found in different insect vector individuals whereas the analysed plant material was more homogenously infected. The established method is now suitable for the study of the infectivity of the insect vectors as well as for the evaluation of the resistance in plant material

INTRODUCTION

A new outbreak of apple proliferation (AP) has been observed in Southwest Germany since 2000. The disease has regained economic importance not only in Germany but also in the great apple growing regions of Northern Italy. For this reason recent research has focused on the prevention of the disease spread by insect vectors and on the development of AP-resistant rootstocks. Recently, the psyllid *Cacopsylla picta* (synonym *C. costalis*) has been identified as vector of AP phytoplasmas in Northern Italy (Frisinghelli et al., 2000) and in Germany (Jarausch et al., 2003a). Other *Cacopsylla* species, namely *C. melanoneura*, may also play a role in the transmission of AP phytoplasmas (Tedeschi et al., 2002). For the study of the infectivity of the insect vectors as well as for the evaluation of the resistance in the plant material quantification of the AP phytoplasmas is needed.

First attempts to quantify AP phytoplasmas in plant tissue have been undertaken by DAPI flourescence microscopy. Kartte and Seemüller (1991) found a wide range of phytoplasma concentrations in experimentally inoculated apple genotypes. Jarausch et al. (1996) studied the differences in concentration of AP phytoplasma strains in micropropagated apple. However, only rough estimations about the concentration of the phytoplasmas can be obtained by this method. A first precise quantification of AP phytoplasmas in plants was achieved by competitive PCR (Berges et al., 2000). Again, a wide range of phytoplasma concentrations was measured in different apple genotypes.

Especially resistant rootstocks had only very low AP phytoplasma concentrations.

Quantitative PCR provides a more straightforeward method for a sensitive and specific quantification of AP phytoplasmas in low titer hosts. The most frequently encountered methods of qPCR are TaqMan™ probes, molecular beacons and SYBR™ Green intercalating dye. In all cases a fluorescent signal is generated during PCR that is captured by an optical unit in realtime. SYBR™ Green technology is the easiest and cheapest method but requires that no artefact bands and primer-dimers are produced in the PCR reaction.

Since the last decade, PCR technology has been employed for the sensitive detection of AP phytoplasmas. Specific primers have been selected in a non-ribosomal DNA fragment of AP phytoplasma (Jarausch et al., 1994). These primers have been used to establish a qPCR assay for the quantification of AP phytoplasmas in plant and insects based on SYBR™ Green technology.

MATERIAL AND METHODS

Plant Material and Phytoplasma Isolates

For the establishment of qPCR with plant material healthy and AP phytoplasma-infected micropropagated apple plants were used. These plants were obtained and propagated as described by Jarausch et al. (1996). A total of 60 individual in vitro plants infected with AP phytoplasmas were used. Four samples of naturally AP-infected apple trees were included in the study. Periwinkle plants infected with the AP phytoplasma strain AT which was transmitted by dodder from apple to periwinkle (referenced in Jarausch et al., 1994) served as controls.

Insect Material

AP phytoplasma-infected specimens of *Cacopsylla picta* were collected in Soutwest Germany as described by Jarausch et al. (2003a, 2003b). A total of 35 individuals which were tested positive by PCR for AP phytoplasma were included in the study. Total DNA extracts from PCR-negative individuals were used in serial dilutions for the standard curve quantification method (see below).

Nucleic Acid Extraction and Phytoplasma Detection by PCR

Total DNA was extracted according to a simplified CTAB extraction protocol (Maixner et al., 1995) either from total in vitro propagated plants or from crude phloem preparations of branches of apple trees or from leaf petioles of periwinkle. The fresh weight of the tissue was weighed out prior to extraction and was adjusted to 0,2 to 0,5 g.

Total DNA from psyllids was extracted with the same method. However, psyllid individuals were homogenized with minipestles in Eppendorf tubes and the buffer volumes were scaled down.

AP phytoplasmas were detected in the DNA extracts using PCR with AP phytoplasma-specific primers AP5/AP4 as described by Jarausch et al. (1994).

Quantitative PCR with SYBR™ Green Technology

AP phytoplasma-specific primers AP5/AP4 and AP3/AP4 (Jarausch et al., 1994) were tested in the qPCR assays. The qPCR mastermix for SYBR™ Green I (Eurogentec, Seraing, Belgium) was used according to the supplier's instructions. PCR reactions were set up in low profile PCR tubes with optical flat caps (Biozym) and qPCR was run and analysed in a DNA Engine Opticon fluorescence detection system (MJ research). Cycle conditions for qPCR were as follows for primers AP3/AP4: 2 min 50°C initial incubation to prevent PCR product carry over by uracil-N-glycosylase (UNG) activity, 10 min 95°C to inactivate UNG and to activate Hot Goldstar DNA polymerase and then 40 cycles of 15 s at 95°C, 30 s at 57°C, 30 s at 72°C and 30 s at 67°C (fluorescence detection step). For primers AP5/AP4 these conditions differed only in the annealing step (15 s at 58°C) and in the elongation step (45 s at 72°C). After the runs a melting curve analysis from 50°C to

90°C was performed.

Quantitatification of AP Phytoplasmas with the Standard-curve Quantification Method

The plasmid pUCI196 which contained the target sequences of primers AP5/AP4 and AP3/AP4 (Jarausch et al., 1994) was taken as standard. The plasmid was purified and the amount of DNA was mesaured. This plasmid stock solution was adjusted that it contained 1 x 10^{10} target copies per µl. From this plasmid stock solution serial 10-fold dilutions were prepared ranging from 1 x 10^{8} target copies per µl to 1 copy per µl. For the quantification of AP phytoplasmas in plant samples the plasmid was diluted in total DNA extracts obtained from healthy micropropagated apple. For the quantification of AP phytoplasmas in insect samples the plasmid was diluted in total DNA extracts obtained from PCR-negativ individuals from *C. picta*. In each qPCR run 1µl of each plasmid dilution was run in a separate tube. The cycle threshold (Ct) values of the dilutions were plotted and verified to gave a linear relationship which served as standard curve. The quality of the standard curve was adjusted manually for each run according to the best linear regression coefficient. Then, the Ct-values of each sample were compared to this standard curve and the copy number in each sample was calculated.

RESULTS AND DISCUSSION

Total DNA extracts of known samples were used to establish the qPCR assay. As plant samples single shoots of AP phytoplasma-infected in vitro plants were used and as insect material AP phytoplasma-infected individuals of *Cacopsylla picta* were used. Both plant and insect samples were selected for the varying signal intensity on agarose gel electrophoresis after conventionel PCR. The AP phytoplasma-specific primers AP5/AP4 and AP3/AP4 (Jarausch et al., 1994) were tested to establish the qPCR assays with SYBR™ Green I dye. Primer pair AP5/AP4 yields a 483 bp fragment with good sensitivity in normal PCR. In qPCR with SYBR™ Green I, however, the signals as measured by the Opticon™ detection system and as checked by agarose gel electrophoresis were significantly weaker with the same samples. A possible explanation is the inhibiting influence of SYBR™ Green I on PCR amplification which becomes more evident with longer PCR products. For this reason the primer pair AP3/AP4 was tested which gives rise to a 162 bp fragment. And indeed, primers AP3/AP4 worked as well in qPCR as in conventional PCR with the same DNA extracts. They yielded a single PCR product with plant and insect samples as it could be verified by melting curve analysis in the Opticon™ detection system (Fig. 1) and by agarose gel electrophoresis. No artefact bands or primer-dimers could be observed. Thus, all fluorescence detection during PCR can be attributed to the AP phytoplasma-specific PCR product.

The quantification of AP phytoplasmas in the samples was done by the standard curve quanatification method. The plasmid pUCI196 (Jarausch et al., 1994) containing the chromosomal fragment of AP phytoplasmas from which the specific primers were derived was used as standard. Serial 10-fold dilutions of the plasmid (1 x 10^{8} copies to 1 copy per µl) were done in total DNA extracts of healthy plants and healthy psyllids, respectively, so that the reaction conditions were equivalent to those of the samples. Interestingly, the detection limits of the same plasmid concentrations differed between dilutions with insect DNA or with plant DNA. Whereas 10 copies were consistently detected in dilutions with insect DNA the detection limit was 100 copies in dilutions with plant DNA. This demonstrates the inhibitory effect of remaining substances of the plant tissue in the DNA extracts. After qPCR, the logarithmic concentrations of the plasmid copies were plotted against the Ct-values of the dilutions in order to verify a linear relationship (Fig. 2). In all runs this linear relationship was consistently high (r^2 = 0,985 – 0,998) so that the results from different runs could be compared. As the chromosomal fragment has been shown to be a non-ribosomal fragment it was admitted that a copy of the plasmid equals a phytoplasma detected in the sample. Phytoplasma detection in the samples and plasmid detection in the dilutions took place under the same reaction

conditions so that the Ct-values could be compared directly. Using this method the absolute concentrations of phytoplasmas in the sample were deduced from the standard curve of each qPCR run.

The reproducibility of the qPCR assay was tested in two variations both for plant and insect samples: the intra-assay reproducibility was tested with triplicates of the same sample in the same run, the inter-assay reproducibility was assessed by running the same sample in 3 different runs at different days. Fig. 3 shows examples of the intra-assay reproducibility of triplicates of plant and insect samples. The data determined for the intra-assay reproducibility of 8 selected insect samples are given in Table 1. Although the reproducibility of the Ct-values is high (coefficient of variation CV = 0,5-4,0%) the deduced copy numbers for each individual vary considerably. The inter-assay reproducibility for the same samples was comparable high with CV-values ranging from 2,2 – 3,1%. The intra- and inter-assay reproducibility of plant samples was tested with 26 AP phytoplasma-infected in vitro plants. The intra-assay reproducibility was as good as with insect samples, the CV-values ranged from 0,1-4,4%. Table 2 shows the data obtained for 3 different runs of the same sample. Again a good inter-assay reproducibility was found with respect to the Ct-values (CV = 0,3-5,8%). However, the deduced copy numbers varied strongly. The absolute quantification is therefore only valuable for a range of phytoplasma concentration in a sample. In *C. picta* individuals phytoplasma titers of 84 to 247.175 bacteria were mesaured. Further studies are now necessary to determine which phytoplasma load is sufficient to render a vector individual infectious. The phytoplasma titers determined in the plant samples were referred to the fresh weight of the extracted plant material. Thus, a maximum of 5,9 x 10^6 phytoplasmas per g fresh tissue of in vitro plants was found (Tab. 2). For comparison, the phytoplasma load of the experimental host plant periwinkle (8,9-9,7 x 10^6 phytoplasmas per g) and of naturally infected apple trees in the orchards (1,1-25 x 10^6 phytoplasmas per g) were determined. These values are in the same range as it was determined for naturally infected trees by competitve PCR (2,7 x 10^6-1,1 x 10^8 phytoplasmas per g; Berges et al., 2000).

In conclusion, a robust, specific and sensitive qPCR assay could be established to quantify AP phytoplasmas in insect and plant material. This assay will now be applied for the study of the infectivity of psyllid vectors and for the evaluation of AP-resistant plant material.

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Tables

Table 1. Intra-assay reproducibility of AP phytoplasma quantification by qPCR in insect samples

Sample	Ct	SD (Ct)	CV % (Ct)	Copies/ individual	SD (copies)
C. picta individual n°3	22	0,11	0,5	247.175	19.269
C. picta individual n°8	22	0,18	0,8	240.095	30.678
C. picta individual n°6	22	0,40	1,8	215.743	64.632
C. picta individual n°7	23	0,58	2,6	160.958	72.149
C. picta individual n°2	24	0,49	2,1	75.807	24.316
C. picta individual n°4	26	1,04	4,0	14.828	8.869
C. picta individual n°1	28	0,74	2,6	3.565	1.867
C. picta individual n°5	33	0,30	0,9	84	19

Table 2. Inter-assay reproducibility of AP phytoplasma quantification by qPCR in plant samples

Sample	Ct	SD (Ct)	%CV (Ct)	Copies	SD (copies)	Copies/ g fresh weight	SD (copies)
S24	24,6	1,4	5,8	217.846	143.514	5.936.000	3.910.000
S21	24,9	1,0	3,8	169.769	64.301	1.812.000	686.000
S1	26,5	0,3	1,1	60.763	15.584	2.319.000	595.000
S11	27,2	0,5	1,9	38.904	5.516	1.926.000	273.000
S20	27,3	1,3	4,7	45.459	26.465	245.000	143.000
S6	27,8	0,7	2,7	26.967	2.253	534.000	45.000
S14	27,9	1,1	4,0	26.618	6.997	924.000	243.000
S2	28,0	0,2	0,7	24.659	8.483	865.000	298.000
S15	28,3	1,6	5,7	21.352	10.586	516.000	256.000
S9	28,6	0,9	3,3	17.062	3.053	126.000	22.000
S23	28,7	1,2	4,3	17.392	8.774	507.000	256.000
S13	29,2	1,0	3,3	11.340	1.928	1.418.000	241.000
S12	29,6	1,2	3,9	9.191	1.939	446.000	94.000
S18	30,4	0,9	2,9	5.847	2.603	40.000	18.000
S26	30,5	1,7	5,6	7.097	5.052	538.000	383.000
S25	30,8	1,5	4,8	4.926	3.176	201.000	129.000
S16	31,6	0,1	0,3	1.882	81	50.000	43.000
S8	31,6	1,0	3,3	2.548	191	154.000	11.000
S4	32,1	0,8	2,4	1.918	215	43.000	5.000
S10	32,9	0,5	1,5	1.162	369	33.000	11.000
S22	33,0	1,6	5,0	1.283	870	36.000	24.000
S3	34,0	1,0	2,9	595	79	24.000	3.000
S19	34,1	1,2	3,5	781	683	13.000	12.000
S7	34,9	0,8	2,4	344	51	9.000	1.000

Figures

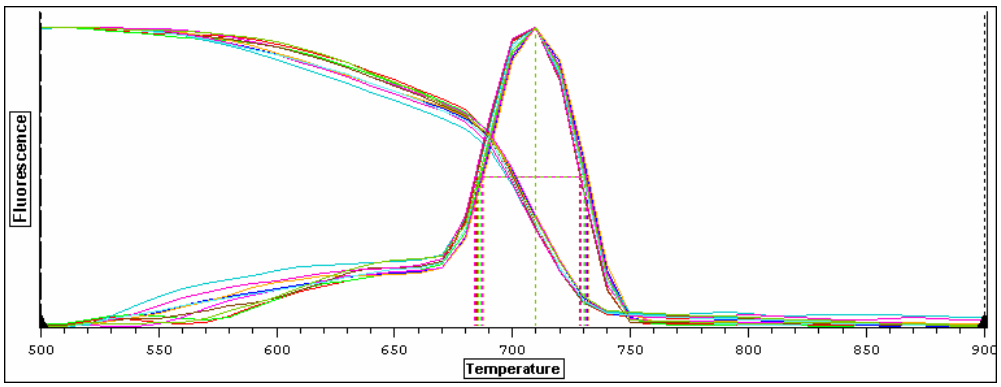

Fig.1. Melting curve analysis of 10 different AP-phytoplasma infected plant samples

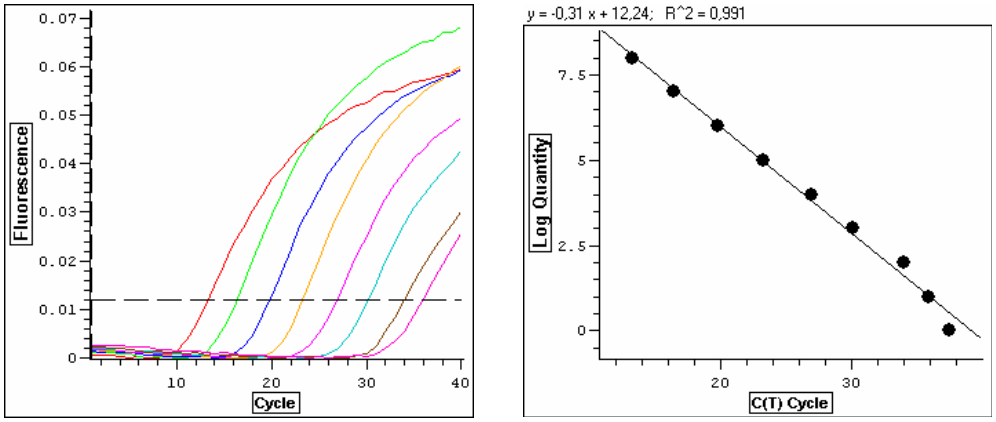

Fig. 2. qPCR with serial 10-fold dilutions of the plasmid as standard (1 x 10^8 copies to 10 copies) and plot of the logarithmic concentrations of the plasmid copies were against the Ct-values

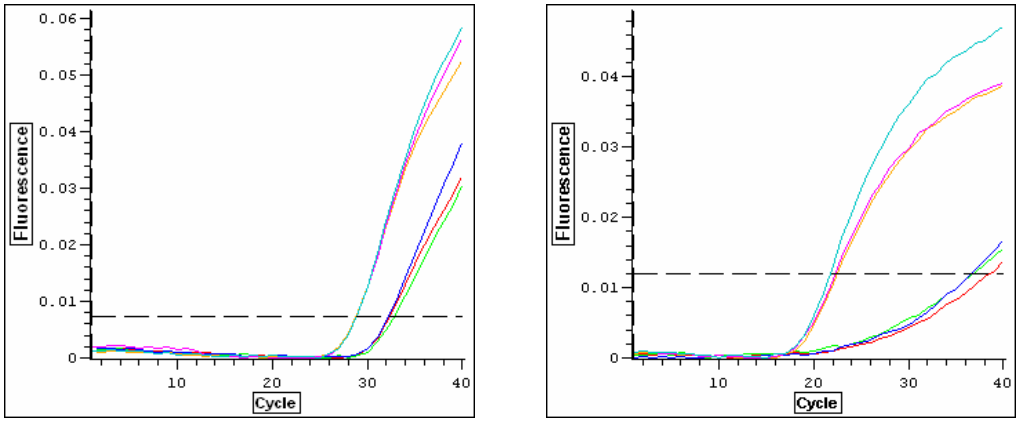

Fig. 3. Intra-assay reproducibility of triplicate samples of plants (left) and insects (right)