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Detection of four apple viruses by multiplex RT-PCR assays with coamplification of plant mRNA as internal control

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Abstract

Two multiplex RT-PCR assays with specific coamplification of plant mRNA as an internal control from total nucleic acids are described for the parallel detection of Apple chlorotic leaf spot virus, Apple stem pitting virus, Apple mosaic virus and Apple stem grooving virus. All are important economically and common pathogens in commercial apple and pear cultivars, except for Apple mosaic virus. Four virus specific primer pairs and one primer pair which allows the specific amplification of mRNA of the mitochondrial *nad*5 gene are described. Specificity of all primer pairs was confirmed by sequencing the RT-PCR products. A range of different virus isolates from various geographic origins could be detected by these multiplex RT-PCR assays all year round. Viruses were detected reliably in composite extracts at a ratio of one part total nucleic acid extract from an infected sample mixed with 39 parts of extract from healthy samples. The use of the internal control minimizes the risk of obtaining false negative RT-PCR results, which is desirable for routine testing, and avoids the need to eliminate contaminating DNA in extracts. To our knowledge, this is the first report on the use of a specific internal RNA control from total nucleic acids. The multiplex RT-PCR assays described are reliable, rapid and sensitive methods for the detection of these viruses, and may replace techniques need commonly like indexing by woody indicators or ELISA.

Keywords: Apple virus detection; Multiplex RT-PCR; Internal control

1. Introduction

Apple (*Malus x domestica* Borkh.) is one of the most widely grown fruit crops worldwide. Apple chlorotic leaf spot virus (*Trichovirus*), Apple stem pitting virus (*Foveavirus*), Apple mosaic virus (*Ilarvirus*) and Apple stem grooving virus (*Capillovirus*) are economically important and common pathogens in commercial cultivars (Mink, 1989a,b; Stouffer, 1989; Welsh and van der Meer, 1989; Desvignes, 1999). Significant yield reduction of up to 60% is possible, especially for frequent mixed infections (Campbell, 1963; Posnette et al., 1963; Schmidt, 1972; Zahn, 1996). Except Apple mosaic virus, infections of these

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viruses in apple remain mostly symptomless in apple and pear cultivars (Nemeth, 1986). For the certification of plant material, apple plants have to be tested, along with other pathogens, for these four distinct virus diseases (EPPO, 1999). To date, woody indicators have been used widely for their detection, although ELISA tests have been developed, but they often fail because of low virus titer and the inhibitory effects of compounds in the sap of woody plants. Furthermore, no antibodies for Apple stem pitting virus are available commercially (Nemchinov and Hadidi, 1998). The use of woody indicators has its disadvantages too: indexing is time consuming (up to 3 years), expensive, and the results may be difficult to interpret.

For routine diagnosis, reliable, fast and inexpensive procedures are essential, therefore, PCR techniques would provide a possible alternative. Using multiplex assays (e.g. Nie and Singh, 2000; Saade et al., 2000; Sharman et al. 2000), the costs and number of samples might be further decreased. To ensure the reliability and facilitate the interpretation of negative results, an internal control would be desirable. Several different internal plant control primers have been published (e.g. Bariana et al., 1995; Pastrik, 2000), but none of them differentiate between RNA and DNA templates, so that complete elimination of DNA is required prior to performing RT-PCR (Nassuth et al., 2000). One alternative is to design primers which amplify different sized fragments due to the presence of introns in the targeted gene, but each additional template will increase the risk of outcompeting the viral templates in the multiplex RT-PCR, and might complicate the identification of the other fragments on the gel. Another technique, reported in this paper, is to design one primer which spans an intron in this manner, so that efficient annealing and amplification is only possible after splicing the intron of the targeted gene. In combination with a second primer positioned in an adjacent exon, this will allow specific amplification of mRNA even in the presence of the genomic DNA, because mismatches at the 3′-end of a primer will inhibit the elongation by polymerases under stringent conditions (Kwok et al., 1990; Innis and Gelfand, 1999), which will appear if the intron is present. Different RT-PCR protocols have been published for the detection of these four important viruses on apple (e.g. Candresse et al., 1995; Rowhani et al., 1995; Kinard et al., 1996; Jelkmann and Keim-Konrad, 1997; James, 1999) but none of them covering all. Furthermore, in these current RT-PCR procedures only pathogen specific primers are utilised. This results in difficulties interpreting negative results because these may be due to RNA degradation or the presence of inhibitors of the reverse transcriptase or polymerase activity, instead of absence of the pathogen. False-negative results are the direct consequence. Often immunocapture techniques are used, making the incorporation of an internal control impossible.

The combination of a simple silica-based nucleic acid extraction with two multiplex RT-PCR assays is described together with specific coamplification of plant mRNA as internal control out of total nucleic acids. All four viruses can be detected from one extract by two identical multiplex RT-PCR assays, each for two viruses and the internal control, only the primers have to be exchanged. Using bark material, the test can be carried out all the year. To our knowledge, this is the first report on a specific internal RNA control out of total nucleic acid extracts, which avoids additional extraction steps to eliminate plant DNA.

2. Materials and methods

2.1. Plant material

Most samples were taken from healthy or infected hosts (species and/or cultivars of *Malus*, *Pyrus*, *Prunus* and *Pyronia*) with single or mixed infections during summer, but some were additionally taken over the whole year. Except for samples W1–W3, the samples were obtained from a field virus collection at the Federal Biological Research Center for Agriculture and Forestry at Dossenheim or the Plant Protection Office of the Chamber of Agriculture at Hanover, Germany. A survey of the virus isolates based on woody indexing according to EPPO recommendations (EPPO, 1999) is given in Table 1, the healthy samples are

Table 1
Host species and geographic origin of virus isolates used for multiplex RT-PCR assays

Sample reference[a]	Host species	Geographic origin	Diagnosed virus(es)[b]
146/80	*Malus x domestica*	Germany	Apple stem grooving virus
40/85	*Malus x domestica*	United Kingdom	Apple stem pitting virus
38/85	*Malus x domestica*	Germany	Apple chlorotic leaf spot virus
41/87	*Malus x domestica*	Germany	Apple mosaic virus, Apple stem grooving virus, Apple chlorotic leaf spot virus, Apple stem pitting virus
31/86	*Pyrus communis*	Germany	Apple stem pitting virus
56/86	*Malus x domestica*	United Kingdom	Apple stem grooving virus, Apple chlorotic leaf spot virus
22/85	*Pyrus communis*	Germany	Apple stem pitting virus
94/87	*Pyrus communis*	Germany	Apple stem pitting virus
33/88	*Malus x domestica*	Germany	Apple stem pitting virus, Apple chlorotic leaf spot virus
35/88	*Malus x domestica*	Germany	Apple stem pitting virus, Apple stem grooving virus, Apple chlorotic leaf spot virus
42/87	*Malus x domestica*	Germany	Apple chlorotic leaf spot virus, Apple stem pitting virus, Apple stem grooving virus
25/88	*Prunus x domestica*	Germany	Apple chlorotic leaf spot virus
4/94	*Malus x domestica*	France	Apple stem pitting virus, Apple stem grooving virus, Apple chlorotic leaf spot virus
33/94	*Malus x domestica*	United Kingdom	Apple stem pitting virus, Apple mosaic virus, Apple chlorotic leaf spot virus
41/94	*Malus x domestica*	Switzerland	Apple stem pitting virus, Apple stem grooving virus, Apple chlorotic leaf spot virus
5/94	*Malus x domestica*	France	Apple stem pitting virus, Apple stem grooving virus, Apple chlorotic leaf spot virus
7/94	*Malus x domestica*	France	Apple stem pitting virus, Apple stem grooving virus, Apple chlorotic leaf spot virus
8/94	*Malus x domestica*	France	Apple stem pitting virus, Apple stem grooving virus, Apple chlorotic leaf spot virus
10/94	*Malus x domestica*	France	Apple stem pitting virus, Apple chlorotic leaf spot virus
18/94	*Malus x domestica*	France	Apple stem pitting virus, Apple stem grooving virus
71/94	*Pyrus communis*	Italy	Apple stem pitting virus
62/94	*Pyrus communis*	Netherlands	Apple stem pitting virus
(1)/96	*Prunus x domestica*	France	Apple chlorotic leaf spot virus
(2)/96	*Prunus avium*	Hungaria	Apple chlorotic leaf spot virus
(3)/96	*Malus x domestica*	Japan	Apple chlorotic leaf spot virus
F 00	*x Pyronia veitchii*	Germany	Apple chlorotic leaf spot virus
W1	*Malus x domestica*	Germany	Apple mosaic virus, Apple stem pitting virus, Apple chlorotic leaf spot virus
W2	*Malus x domestica*	Germany	Apple mosaic virus, Apple stem pitting virus, Apple stem grooving virus, Apple chlorotic leaf spot virus
W3	*Malus x domestica*	Germany	Apple mosaic virus, Apple stem grooving virus, Apple chlorotic leaf spot virus

[a] Samples in bold letters were only pre-tested for Apple stem pitting virus.
[b] Except samples W1, W2 and W3 the disease status was determined by woody indicators at the Federal Biological Research Center for Agriculture and Forestry at Dossenheim or the Plant Protection Office of the Chamber of Agriculture at Hanover, Germany. By multiplex RT-PCR additionally detected infections are underlined.

not listed. For testing the suitability of the internal control, samples of *Nicotiana benthamiana*, *Nicotiana tabacum* and *Chenopodium quinoa* were also taken. The samples were stored in plastic bags at 4 °C until extraction.

2.2. Extraction of total nucleic acids

Total nucleic acids were extracted according to a modified method described originally by Boom et al. (1990) for the extraction of nucleic acids from serum, and adapted for the detection of plant viruses from woody plants by MacKenzie et al. (1997) and Rott and Jelkmann (2001). Instead of leaf material, which was only taken for herbaceous plants and for additional extracts for some samples, about 100 mg of bark chips (3–5) containing vegetative buds from the basis of 1–2-year-old branches were ground in a mortar with 1 ml grinding buffer (6 M guanidine hydrochloride instead of 4 M guanidine thiocyanate, 0.2 M sodium acetate pH 5.2, 25 mM EDTA, 1 M potassium acetate and 2.5% PVP-40). The replacement of guanidine thiocyanate by guanidine hydrochloride avoids the risk of forming prussic acid and 6 M guanidine hydrochloride is as efficient in inactivating ribonucleases and dissociating nucleoproteins into its nucleic acids and protein moieties as 4.5 M guanidine thiocyanate (Lepanje, 1971; MacDonald et al., 1987). In comparison to the original papers no ß-mercaptoethanol was added to the grinding buffer, which did not influence the amplification as ascertained in parallel tests (not shown), so eliminating the need to use a fumehood. Five hundred microlitres of the homogenate was transferred to a 2 ml safe-lock Eppendorf-tube to which 100 µl SDS (10%) was added. The mixture was incubated at 70 °C for 10 min with intermittent shaking, and placed on ice for 5 min. It was then centrifuged at 13 K for 10 min. Three hundred µl of the supernatant was transferred to a new tube and 300 µl sodiumiodide-solution (6 M, stabilized by 0.15 M sodium sulfite), 150 µl ethanol (99.6%) and 25 µl silica-suspension (1 g/ml silica particles (Sigma S5631), pH 2) were added. The mixture was incubated at room temperature for 10 min with intermittent shaking and centrifuged for 1 min at 6 K. The supernatant was removed and the pellet resuspended in 500 µl wash buffer (10 mM Tris–HCl pH 7.5, 0.05 mM EDTA, 50 mM NaCl and 50% ethanol). The wash step was repeated and the pellet was resuspended in 150 µl TE buffer (10 mM Tris–HCl pH 7.5, 0,1 mM EDTA) and incubated at 70 °C for 4 min to redissolve the nucleic acids. After a final centrifugation step of 5 min at 13 K, the supernatant was transferred to a new tube and stored at −20 °C. In order to determine the detection limits of the multiplex assays, total nucleic acid extracts of fourfold infected samples were diluted serially in total nucleic acid extract from healthy control plants (1:2, 5, 10, 20, 40, 100, 200, 500 and 1000), and used in multiplex RT-PCR assay I and II. Diluting the total nucleic acid extract of the infected sample in total nucleic acid extract of a healthy control plant will lower the concentration of virus templates but not of the *nad*5 mRNA template of the internal control primers.

2.3. Internal control

Kato et al. (1995) characterised a part of the mitochondrial DNA from apple and described the existence of the *nad*5 gene (NADH dehydrogenase subunit 5) containing the exons a and b, separated by an 848 bp intron. The sense primer (*nad*5-s) of the internal control was designed in this manner, that the last three nucleotides at the 3′-end are homologous to the first three nucleotides of exon b and the complete 5′-tail of this primer is homologous to the 5′-end of exon a. The antisense primer (*nad*5-as) was positioned entirely in exon b (Fig. 1). In order to control the exclusive amplification of RNA even if genomic DNA was present, controls of RT-PCR were performed in the absence of reverse transcriptase, and DNase treated total nucleic acid extracts were also tested. For these assays the internal control primers were used solely at a higher concentration in the RT-PCR mixture (0.1 µM instead of 0.04 µM). The primer sequences are given in Table 2.

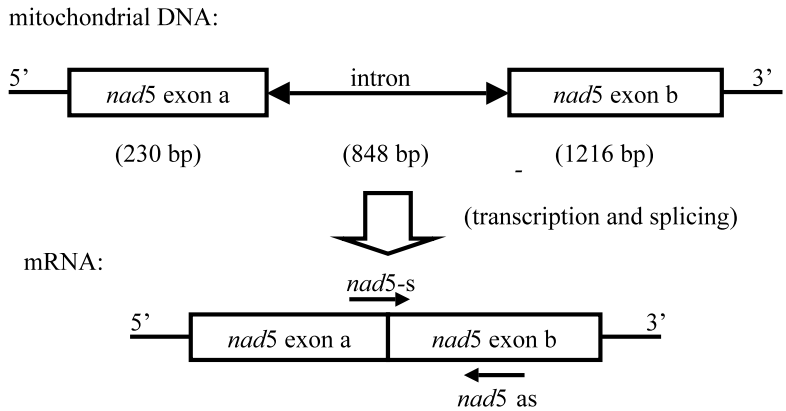

Fig. 1. Schematic representation of the location of the internal control primers (indicated by arrows) in the mitochondrial *nad*5 gene. Segments are not drawn to scale.

2.4. Virus specific primers

Highly conserved regions were determined from sequence alignments of all available sequences from the GenBank of the National Center for Biotechnology Information for each virus (Apple chlorotic leaf spot virus: M58152, AJ243438, D14996, X99752; Apple stem pitting virus: D21829, D21828; Apple mosaic virus: U15608, L03726, S78319; Apple stem grooving virus: D16681, AB004063, D16368, D14455, AF233298, AF233299, D14995) and additional sequence information given in the literature (Apple chlorotic leaf spot virus: Kinard et al., 1996; Malinowski et al., 1998; Apple stem pitting virus: Schwarz and Jelkmann, 1998; Apple mosaic virus: Candresse et al., 1998). Primers for these regions were then designed based on the recommendations of Innis and Gelfand, 1999), using the program Oligo 4.0 (Rychlik and Rhoads, 1989). The alignments were carried out with the program ClustalX 1.64b. In Table 2 the primer sequences, primer positions and expected amplified fragment size are given for each template.

2.5. Multiplex RT-PCR assay I and II

The multiplex RT-PCR assays were optimized according to Henegariu et al. (1997) and carried out in a T3 thermal cycler (Biometra). The reaction mixture contained 1 μl of total nucleic acid extract, 7.5 μl 10 × PCR buffer (provided with the HotStar *Taq* polymerase, QIAGEN), 0.5 μl $MgCl_2$ (25 mmol/l), 0.5 μl Tween-20 (10%), 0.5 μl dNTP mixture (each dNTP 25 mmol/l), 0.05 μl AMV reverse transcriptase (10 U/μl, Promega), 0.4 μl HotStar *Taq* polymerase (5U/μl, QIAGEN), 0.2 μl of each *nad*5 primer (10 μmol/l). For multiplex I (detection of Apple chlorotic leaf spot virus and Apple stem grooving virus) the mixture contained additionally 1.5 μl of each Apple chlorotic leaf spot virus primer (10 μmol/l) and 1.75 μl of each Apple stem grooving virus primer (10 μmol/l) and for multiplex II (detection of Apple mosaic virus and Apple stem pitting virus) 1.5 μl of each Apple mosaic virus primer (10 μmol/l) and 0.75 μl of each Apple stem pitting virus primer (10 μmol/l). Sterile deionized water was added to a final volume of 50 μl. The cycling parameters were: reverse transcription at 42 °C for 30 min, activation of the HotStar *Taq* polymerase at 95 °C for 15 min followed by 34 cycles of denaturation at 94 °C for 30 s, annealing at 62 °C for 30 s, and extension at 72 °C for 1 min. The final extension step was 72 °C for 7 min. The reaction mixture was set up in a separate room on a clean-bench which was decontaminated by ultraviolet light in order to prevent contaminations according to the recommendations of Singh (1998). To ensure the absence of contaminations, each RT-PCR run included a negative (water) control. If required, additional positive controls were also included.

2.6. Analysis of amplified products

Aliquots (10 μl) of the products were electrophoresed in 1.5% agarose gels in 1 × TAE buffer (40 mmol/l tris–acetate, 1 mmol/l EDTA) for 1 h at 120 V and stained with ethidium bromide. The gels were visualized by ultraviolet light illumination at 312 nm. Sizes of fragments were determined by comparison with *Pst*I digested λ-DNA as molecular weight marker. In order to confirm the identity of the amplified products, fragments of each virus and the internal control were purified from the agarose gels with QIAEX II Gel Extraction Kit (Qiagen). These fragments were ligated into the vector pBluescriptII-SKM (Stratagene), cloned into *Escherichia coli* NM 522 (Pharmacia) and sequenced.

3. Results

3.1. Extraction

Extraction and multiplex RT-PCR were carried out all the year round. Using bark chips containing vegetative buds for extraction, the test worked well even for the samples taken in the dormant season in winter. Several samples were stored at 4 °C for up to 8 weeks without any obvious negative effect on the subsequent detection by multiplex RT-PCR of the viruses and the internal control. For some samples, additional extracts were made using fully developed, young leaves. This resulted, in most cases, in an obvious reduction in band intensity of RT-PCR products on the gel, for some Apple mosaic virus isolates no product could be detected using leaf material (data not shown).

3.2. Internal control

The designed primer pair for the internal control amplified consistently a RT-PCR fragment of the expected size (181 bp), irrespective of whether the extract came from infected or healthy plants. No differences could be observed between the varying host species which were used (Table 1). For all different species, tests were also undertaken in the absence of reverse transcriptase and with DNase

Table 2
Primer sequences and expected size of RT-PCR product for each primer pair

Primer	Primer sequence in 5′–3′ orientation	Primer position[a]	Product size
Apple chlorotic leaf spot virus			
Sense	TTCATGGAAAGACAGGGGCAA	6860–6880, 7507–7536	677 bp
antisense	AAGTCTACAGGCTATTTATTATAAGTCTAA		
Apple stem pitting virus			
Sense	ATGTCTGGAACCTCATGCTGCAA	8869–8895, 9211–9238	370 bp
antisense	TTGGGATCAACTTTACTAAAAAGCATAA		
Apple mosaic virus			
Sense	ATCCGAGTGAACAGTCTATCCTCTAA	1474–1499, 1711–1735	262 bp
antisense	GTAACTCACTCGTTATCACGTACAA		
Apple stem grooving virus			
Sense	GCCACTTCTAGGCAGAACTCTTTGAA	6039–6064, 6286–6311	273 bp
antisense	AACCCCTTTTTGTCCTTCAGTACGAA		
nad5			
Sense	GATGCTTCTTGGGGCTTCTTGTT	968–987 and 1836–1838,	181 bp
antisense	CTCCAGTCACCAACATTGGCATAA	1973–1995	

[a] The reference accession numbers (National Center for Biotechnology Information) for the determination of the primer positions are D14996 for Apple chlorotic leaf spot virus, D21828 for Apple stem pitting virus, U15608 for Apple mosaic virus, AB004063 for Apple stem grooving virus and D37958 for the nad5 gene.

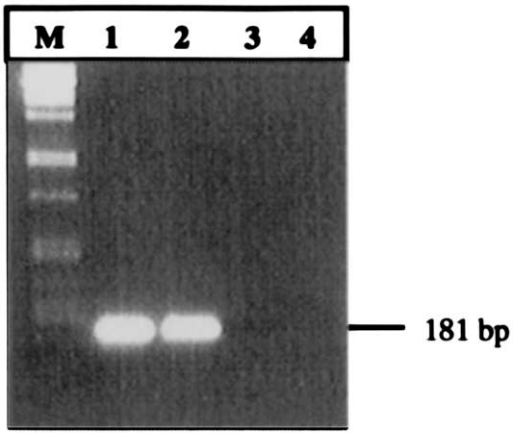

Fig. 2. Agarose gel analysis of RT-PCR assays on the internal control using total nucleic acid extract (lanes 1 and 3) and DNase treated total nucleic acid extract (lane 2 and 4) in the presence (lanes 1 and 2) or absence (lanes 3 and 4) of reverse transcriptase. Size marker (lane M): *Pst*I digested λ-DNA.

treated total nucleic acid extracts. As expected, in the presence of reverse transcriptase a corresponding fragment could be amplified for total nucleic acid and RNA extracts (Fig. 2). Without reverse transcriptase, amplification of the internal control was not observed. Even for the herbaceous plants (*N. benthamiana*, *N. tabacum* and *C. quinoa*) the primers amplified a fragment of the expected size and discriminated between RNA and DNA as template. The amplification of the endogenous template had to be limited, otherwise it would probably outcompete the viral templates. Therefore, the concentration of the internal control primers was lowered to 0.04 μM in the multiplex RT-PCR.

3.3. Detection limits of multiplex RT-PCR assay I and II

Total nucleic acids extracted from bark material in summer of fourfold infected samples were serially diluted in total nucleic acid extracts from healthy control plants and used for multiplex RT-PCR I and II. For multiplex RT-PCR I, specific bands could be detected for Apple chlorotic leaf spot virus and Apple stem grooving virus up to 1:40 dilution (Fig. 3) and applying multiplex RT-PCR II Apple stem pitting virus could be detected up to 1:40 and Apple mosaic virus up to 1:100 dilution (Fig. 4). As expected, a specific RT-PCR product of the internal control could be detected in all dilutions for multiplex assay I and II without any obvious difference in band intensity on the gel.

3.4. Amplification of selected isolates by multiplex RT-PCR

To test whether multiplex RT-PCR assays I and II are suitable and reliable for diagnosis of Apple

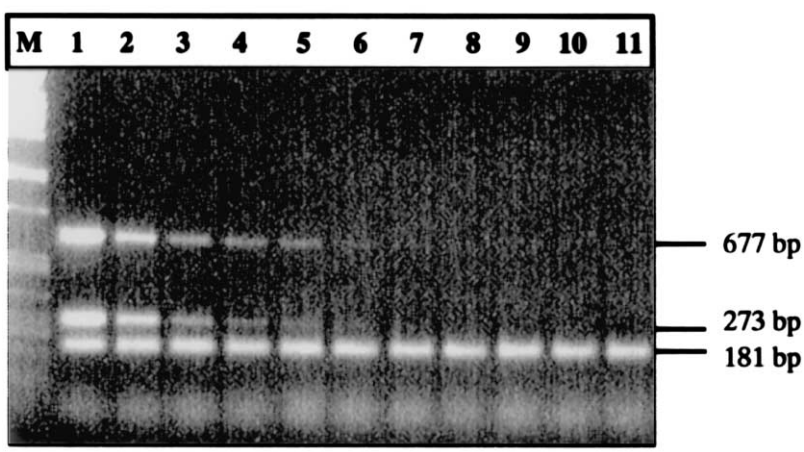

Fig. 3. Estimation of the detection limit of multiplex RT-PCR I for Apple chlorotic leaf spot virus and Apple stem grooving virus. Size marker (lane M): *Pst*I digested λ-DNA. Lane 1, RT-PCR products of undiluted total nucleic acid extract of a fourfold infected sample; lane 2–10, RT-PCR products of total nucleic acid extract of a fourfold infected sample serially diluted in total nucleic acid extract of a healthy control plant (1:2, 5, 10, 20, 40, 100, 200, 500, 1000); lane 11, RT-PCR product of total nucleic acid extract of the healthy control.

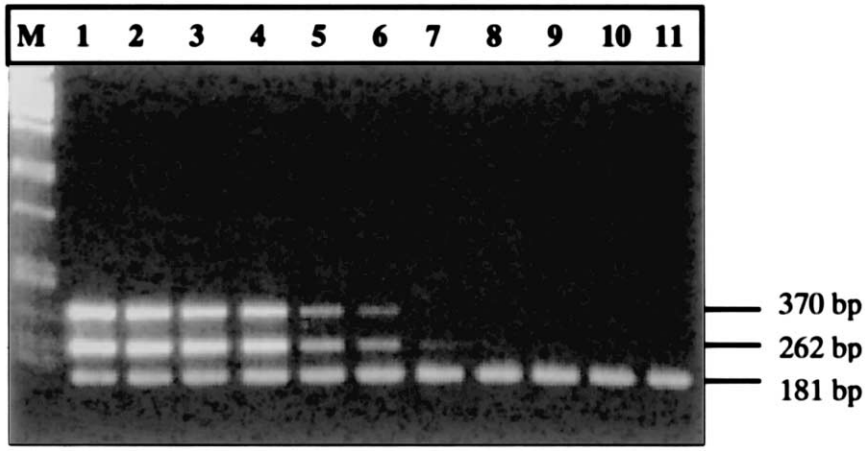

Fig. 4. Estimation of the detection limit of multiplex RT-PCR II for Apple stem pitting virus and Apple mosaic virus. Size marker (lane M): *Pst*I digested λ-DNA. Lane 1, RT-PCR products of undiluted total nucleic acid extract of a fourfold infected sample; lane 2–10, RT-PCR products of total nucleic acid extract of a fourfold infected sample serially diluted in total nucleic acid extract of a healthy control plant (1:2, 5, 10, 20, 40, 100, 200, 500, 1000); lane 11, RT-PCR product of total nucleic acid extract of the healthy control.

chlorotic leaf spot virus, Apple stem grooving virus, Apple stem pitting virus and Apple mosaic virus in apple, several isolates in single or mixed infections of different geographic origin were tested (Table 1). Additionally, the assays were evaluated by some virus isolates of varying hosts (species of *Prunus*, *Pyrus* and *Pyronia*). In all assays, healthy control plants and negative controls were included. Healthy samples from all species tested produced a single band of 181 bp derived from the internal control template. None of the negative controls ever produced a detectable RT-PCR fragment. Examples for multiplex RT-PCR I and II are given in Figs. 5 and 6. The multiplex assays amplified all virus isolates tested in this study as expected from woody indexing results (Table 1). The band intensity of the individual fragments on the gel varied between the different viruses and virus isolates and also varied for different total nucleic acid extracts of the same plant (not shown), but all infections of the pre-tested samples could be always confirmed. For solely Apple stem pitting virus pre-tested samples, additional infections could be detected (Table 1). For all Apple chlorotic leaf spot virus infected samples, in multiplex RT-PCR assay I, an additional faint fragment of about 600 bp could be observed on the gel, indicated by an arrow in Fig. 5.

During development of the multiplex RT-PCR assays, mixtures of three virus specific primer pairs (without internal control) for the simultaneous amplification of Apple chlorotic leaf spot virus, Apple stem pitting virus and Apple mosaic virus were also tested. This resulted in strong differences in band intensity of the specific fragments on the agarose gel for total nucleic acid extracts of plants infected with these three viruses (not shown). In some cases, single bands could not be observed, resulting in false negative results for the corresponding virus. In contrast, by the combination of two viruses and the internal control (multiplex RT-PCR assay I and II) the differences in band intensity were slight and no false negative results appeared. All expected fragments could be clearly identified.

3.5. Sequence analysis of RT-PCR products

Sequence comparison of cloned amplified fragments of each virus and the internal control with the published sequences at the GenBank (National Center for Biotechnology Information) resulted in sequence identities from 82 to 98% (Apple chlorotic leaf spot virus, 82–93%; Apple stem pitting virus, 86–87%; Apple mosaic virus, 94–96%; Apple stem grooving virus, 90–91% and *nad*5, 98%).

4. Discussion

This paper describes two identical multiplex RT-PCR assays (only virus specific primers have to be exchanged) for the parallel detection of four economically important and widespread viruses of apple and the specific coamplification of plant mRNA as internal control. Methods other than RT-PCR can be used for detecting these viruses, but they are labor-intensive, expensive and tedious (woody indicators) or not suitable (ELISA) (Nemchinov et al., 1995; Kinard et al., 1996; MacKenzie et al., 1997). An internal control procedure is lacking for immunocapture RT-PCR and ELISA tests (Sharman et al., 2000), making them unsuitable for routine testing if false negative results caused by e.g. inhibitors have to be excluded. Previously described RT-PCR or multiplex RT-PCR assays do not cover all four viruses and no internal control is included, making the interpretation of negative results difficult. We have developed two multiplex RT-PCR assays including an internal control which offer an attractive alternative. The total nucleic acid extraction procedure described was modified in order to exclude highly toxic constituents like guanidine thiocyanate and ß-mercatoethanol, eliminating the need of a hood for extraction. This will facilitate the portability of this procedure to laboratories where routine tests are carried out and minimizes the health risk for the staff. Using bark chips with vegetative buds, the test could be conducted all the year round, whereas ELISA or indexing by woody indicators (grafting) can only be performed at the appointed time (Candresse et al., 1995; Kinard et al., 1996). Results of multiplex RT-PCR using total nucleic acid extracts of fully developed leaves were unsatisfactory, the band intensity of the RT-PCR products on the gel was often obviously reduced. This might be due to lower virus titer in leaves and/or inhibitory effects of polysaccharides or phenolic compounds on reverse transcriptase and/or *Taq* polymerase (Demeke and Adams, 1992; Staub et al., 1995 Pandey et al., 1996), which are highly concentrated in apple leaves (Schmitz and Noga, 2000) and might be still present in total nucleic acid extracts. The use of woody plant material is in addition advantageous compared with leaf material due to the simpleness of handling and storage of defoliated branches. It also allows testing of defoliated nursery material when traded. Non DNase-treated RNA extracts are often DNA contaminated (Nassuth et al., 2000), making the use of previously described internal control primers as RT-PCR control difficult for such extracts, because they do not distinguish between RNA and DNA. Either a product of the same size will be amplified from RNA and DNA or differently sized fragments will be amplified, which is due to the presence of an

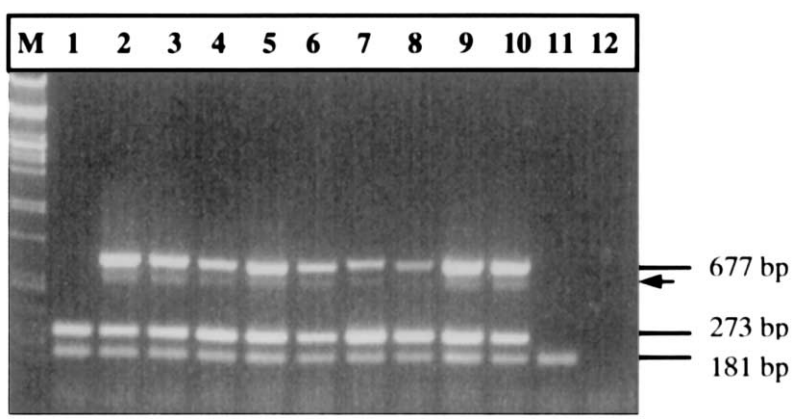

Fig. 5. Representative results for the detection of Apple chlorotic leaf spot virus and Apple stem grooving virus by multiplex RT-PCR I. Size marker (lane M): *Pst*I digested λ-DNA. Lane 1–10, RT-PCR products of various infected samples (146/80, 56/86, 41/94, 35/88, 5/94, 7/94, 8/94, 41/87, 42/87, W2); lane 11, RT-PCR product of a healthy control plant; lane 12, negative control. The additonal fragment of Apple chlorotic leaf spot virus infected samples is indicate by an arrow.

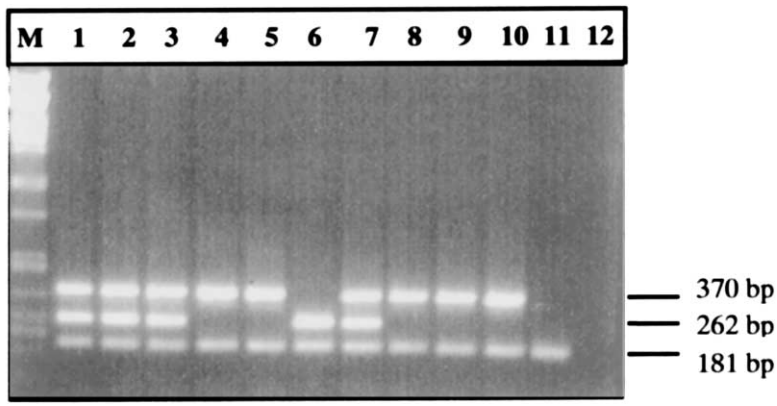

Fig. 6. Representative results for the detection of Apple stem pitting virus and Apple mosaic virus by multiplex RT-PCR II. Size marker (lane M): *Pst*I digested λ-DNA. Lane 1–10, RT-PCR products of various infected samples (41/87, 33/94, W1, 40/85, 31/86, W3, W2, 4/94, 41/94, 71/94); lane 11, RT-PCR product of a healthy control plant; lane 12, negative control.

intron in the corresponding gene, but each additional template in multiplex RT-PCR will compete with the viral templates and could result in difficulties identifying other amplified fragments on the gel. DNase treatment of the extracts will eliminate these problems, but each additional extraction step is time consuming, will raise costs and increases the risk of contaminations. In order to avoid this, a primer pair was designed which allows the specific amplification of mRNA even in the presence of genomic DNA. The internal control was utilized successfully for all different woody and herbaceous plants used in this work. Total nucleic acid and DNase treated total nucleic acid extracts of all different species were tested in the presence and absence of reverse transcriptase to verify the exclusive amplification of RNA and exclude the existence of amplifiable copies of the *nad*5 gene without intron (e.g. pseudogenes). The internal control primers could be successfully used with extracts made all the year, indicating that the mRNA of the corresponding gene is present even in the dormant season, which would be expected, because the product of the *nad*5 gene belongs to the basic metabolism of plants (Heldt, 1997). This is important if the multiplex RT-PCR assays have to be utilized independent from the growing season. The internal control primers may be also suitable for further pathogen-host combinations.

In our experiments with a range of different virus isolates of various geographic origins we were able to detect all in accordance with the pretests by woody indicators. The primers amplified all isolates tested and verified their efficiency. The intensity of the amplified products on the gel was not always the same, also for different total nucleic acid extracts of the same plant, probably as a result of varying virus (template) concentrations in the total nucleic acid extracts, but all fragments could be clearly identified and assigned to the corresponding template by comparison with the size marker. The detection limits of multiplex RT-PCR assay I and II were determined by diluting serially total nucleic acid extract from fourfold infected samples in total nucleic acid extract from healthy control plants. Specific bands on the gel could be detected for Apple chlorotic leaf spot virus, Apple stem grooving virus and Apple stem pitting virus up to 1:40 dilution and for Apple mosaic virus up to 1:100 dilution, corresponding to 2.5 mg and 1 mg of infected tissue in 100 mg in total. This allows detection of the infections of one positive sample out of a bulked extract of 40, which could be useful for large scale indexing in order to decrease costs. However, it will make the results of the internal control worthless, because if the RNA of one infected sample is degraded and, therefore, not amplifiable, the mRNA of the other samples will serve as a template for the internal control primers and false negative results might be the consequence. Composite samples for total nucleic acid extrac-

tions rather than combining total nucleic acid extracts can be used alternatively, but it is very difficult to obtain bark chips of similar size for bulk samples. All primers used in this study were designed to amplify their target sequence under the same RT-PCR cycling conditions, allowing to run multiplex RT-PCR assay I and II in parallel. Also the same total nucleic acid extract and RT-PCR reaction mixture can be used, only the virus specific primers have to be exchanged. The additional amplified fragment (about 600 bp) which could be observed on the gels for Apple chlorotic leaf spot virus infected samples is presumably from mispriming of one or both virus specific primers on the viral template, because it could be also observed if these primers were used alone (not shown) and it occurs only for Apple chlorotic leaf spot virus infected samples. Nevertheless it does not influence the identification of the other fragments on the gel because of its size, and can, therefore, be tolerated. The above described method allows the reliable detection of Apple chlorotic leaf spot virus, Apple stem grooving virus, Apple mosaic virus and Apple stem pitting virus within 1 day (2–4 h extraction of 12–48 samples, 3 h multiplex RT-PCR, 1 h electrophoresis) whereas the at present widely used detection by woody indicators requires a minimum of 12 weeks, up to 3 years (EPPO, 1999). Therefore, the multiplex RT-PCR assays should help to save time and costs in testing for these viruses. In summary, the multiplex RT-PCR assays I and II described here could be a reliable and suitable tool for indexing apple plants for Apple chlorotic leaf spot virus, Apple stem grooving virus, Apple stem pitting virus and Apple mosaic virus, and may replace the presently used less advantageous techniques like ELISA or woody indicators for the indexing of these pathogens.

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