The Application of RT-PCR Assay for the Detection of *Apple Stem Pitting Virus* and *Apple Stem Grooving Virus* in Four Apple Cultivars

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Abstract

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The reverse transcription polymerase chain reaction (RT-PCR) assay was successfully used for the detection of *Apple stem pitting virus* (ASPV) and *Apple stem grooving virus* (ASGV) in four apple cultivars of a 25 years old orchard. These two main pome fruit viruses were detected frequently in all tested apple cultivars. ASGV and ASPV occurred in as many as 16 trees (in the cultivar Spartan) and 13 trees (in the cultivar Idared) out of 20 tested trees, respectively. Mixed infection by ASGV and ASPV was found in all tested cultivars (as many as 9 out of 20 tested trees of the cultivar Spartan).

Keywords: RT-PCR; ASPV; ASGV; *Apple stem pitting virus*; *Apple stem grooving virus*

Apple stem pitting virus (ASPV) and *apple stem grooving virus* (ASGV) are widely distributed in apple and pear growing orchards (NEMETH 1986). ASPV is the member of the genus *Foveavirus* (MARTELLI & JELKMANN 1998) with flexuous filamentous particles 800 nm long and 12–15 nm wide, which have a strong tendency to form end-to-end aggregates (KOGANEZAWA & YANASE 1990). ASGV is the member of the genus *Capillovirus* with flexuous filamentous particles with a clear modal length of 600–700 nm and 12 nm wide (MURPHY *et al*. 1995). ASPV and ASGV cause a disease associated with tree decline, stem pitting and vein yellows (ASPV), and graft union necrosis (ASGV) in apples and pears (WELSH & UYEMOTO 1980; DESVIGNES *et al*. 1990).

The traditional biological virus testing is still time-consuming and of low sensitivity (STOUFFER & FRIDLUND 1989) in spite of the development of some effective *Malus* clones, including *Malus* MO-65 (YANASE & YAMAGUCHI 1982) and *M. micromalus* GMAL273.a (HOWELL *et al*. 1996) for the detection of ASPV and ASGV respectively. ELISA-based techniques are very useful for routine detection of ASGV (FUCHS 1981), but the virus may escape detection because of low concentration (KINARD *et al*. 1996). Furthermore, ASPV cannot be detected by ELISA since no good antiserum is available. The analyses of the nucleotide sequence of the ASPV genome (JELKMANN 1994) and ASGV genome (YOSHIKAWA *et al*. 1992) has allowed the development of techniques based on reverse transcription polymerase chain reaction (RT-PCR) for the detection of these viruses (MACKENZIE *et al*. 1997). Recently, RT-PCR has been widely used for the detection of ASPV (SCHWARZ & JELKMANN 1998; KUMMERT *et al*. 1998; KUNDU 2001) as well as ASGV (KINARD *et al*. 1996; KUMMERT *et al*. 1998). To facilitate the simultaneous detection of both viruses, immunocapture/RT-PCR for ASPV (JELKMANN & KEIM-KONRAD 1997) and ASGV (JAMES 1999) has also been used. Further, some simplified RT-PCR protocols have been developed for the detection of ASGV involving the use of crude sap extracts as template for RT-PCR (MARINHO *et al*. 1998) and tube capture RT-PCR (JAMES 1999).

The occurrence of ASPV in apple orchards in the Czech Republic was proven by biological assay (SEIDL *et al*. 1979) and laboratory assay (KUNDU 2001). ELISA-based tests reported an incidence of over 50% of ASGV in some orchards (JANEČKOVÁ & PLUHAŘ 1987; POLÁK & ZIEGLEROVÁ 2001). This paper describes the use of RT-PCR assay to determine ASPV and ASGV incidence and the frequency of mixed infection by these viruses in four apple cultivars.

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MATERIALS AND METHODS

Virus isolates

ASPV apple isolate (PRI, Wageningen, The Netherlands) was maintained and propagated on *Nicotiana occidentalis* 37B, and the ASGV apple isolate (Aschersleben, Germany) on *Chenopodium quinoa*. These isolates were used as positive controls.

Tested plants from apple orchards

Twenty trees of each of the apple cultivars Idared, Spartan, Vista Bella and Stark Earliest were tested. The trees were grown in a 25 years old orchard (located in Horoměřice, Central Bohemia); there had been no artificial infection. The orchard was chosen according to previous experience with ELISA-based detection of ASGV incidence in it (POLÁK & ZIEGLEROVÁ 2001).

Design of primers for RT-PCR

Two pairs of specific ASPV primers and two pairs of specific ASGV primers were selected from the RNA sequence of the ASPV genome (JELKMANN 1994) and the ASGV genome (YOSHIKAWA *et al*. 1992), respectively, as described in Table 1.

Extraction of total RNA

Total RNA was prepared according to KUNDU (2001) as follows: the leaves of apple (*Malus domestica* Borkh*)* or indicator hosts (*Nicotiana occidentalis* 37B for ASPV or *Chenopodium quinoa* for ASGV) were ground in a mortar and homogenised in extraction buffer (1:10, 20 mM Tris-HCl pH 7.8 containing 200 mM LiCl, 20 mM EDTA and 1% SDS). The extract was centrifuged at 10 000 rpm for 5 min, 500 µl of supernatant were removed, 5 µl of proteinase K (20 mg/ml) added and incubated at 37°C for 1 h. The homogenate (500 µl) was mixed with an equal volume of phenol/chloroform (1:1) for 10 min by vortexing, and centrifuged at 10 000 rpm for 10 min. The aqueous phase was removed and the process was repeated twice. The aqueous phase was then mixed with a triple volume of ice-cold ethanol and 2 M LiCl and the mixture was stored at –20°C for 45 min to precipitate nucleic acids. The precipitate was collected by centrifugation at 14 000 rpm for 10 min and washed two times for 10 min in 70% ethanol at room temperature. Then it was again centrifuged at 14 000 rpm for 10 min, the pellet was dried under vacuum and resuspended in 50 µl of sterile deionised diethyl pyrocarbonate (DEPC) treated water and stored at –25°C until used.

Reverse transcription polymerase chain reaction (RT-PCR)

Synthesis of cDNA

Synthesis of cDNA was performed as follows: 2 µl of extracted RNA was diluted to 10 µl with DEPC treated water and 10 pmol of downstream primer (ASP-A or ASP-II; or ASGV-2 or ASGV-4R). Incubation at 70°C for 5 min and chilling on ice followed. The RT reaction mixture consisting of 5.25 µl of RNAase-free water, 300 units of M-MLV reverse transcriptase, 5 µl of buffer for M-MLV reverse transcriptase, 20 units of RNAsin and 0.2 mM of each dNTP (all from Promega). The reaction mixture was incubated at 37°C for 1 h and then heated at 100°C for 5 min.

Amplification of cDNA

The PCR reaction mixture was prepared in microtubes and consisted of 2.5 µl of buffer for *Taq* polymerase, 2.5 units of *Taq* polymerase, 10 pmol of upstream and downstream primers, 0.2 mM dNTPs and 1.25 mM $MgCl_2$ (all from Promega). The mixture was adjusted to 23 µl with sterile deionised water and 2 µl of cDNA was added

Table 1. Primers

Virus	Primer	Sequence	Nucleotides	Product size	Reference
ASPV	ASP-C[a]	5′-CTCTTGAACCAGCTGATGGC-3′	8993–9012	264 bp	JELKMANN & KEIM-KONRAD (1997)
	ASP-A[b]	5′-ATAGCCGCCCCGGTTAGGTT-3′	9237–9256*		
	ASP-I[a]	5′-AAGAGAAGACATCCAGATTTG-3′	358–378		
	ASP-II[b]	5′-CTATAGCCTCTCCCTTGGT-3′	892–910*	553 bp	KUNDU (2001)
ASGV	ASGV-U[a]	5′-CCCGCTGTTGGATTTGATACACCTC-3′	5873–5897	499 bp	JAMES (1999)
	ASGV-2[b]	5′-GGAATTTCACACGACTCCTAACCCTCC-3′	6345–6371*		
	ASGV-4F[a]	5′-GTTCACTGAGGCAAAAGCTGGTC-3′	3918–3940	574 bp	KUMMERT *et al*. (1998)
	ASGV-4R [b]	5′-GACGACACCTTCTCCATGCCTTC-3′	4469–4491*		

a = Upstream primer
b = Downstream primer (reverse primer)
* = Complementary to nucleotides

into each reaction tube. The reaction was carried out in a thermocycler (MJ Research) as follows:

ASPV: 40 cycles of 94°C for 45 s (denaturation) then 55°C for 1 min (for primer pair ASP-A/ASP-C) or 62°C for 45 s (for primer pair ASP-I/ASP-Il) (annealing) and 72°C for 1 min (polymerisation), and after the last cycle a final 5 min elongation step at 72°C was added;

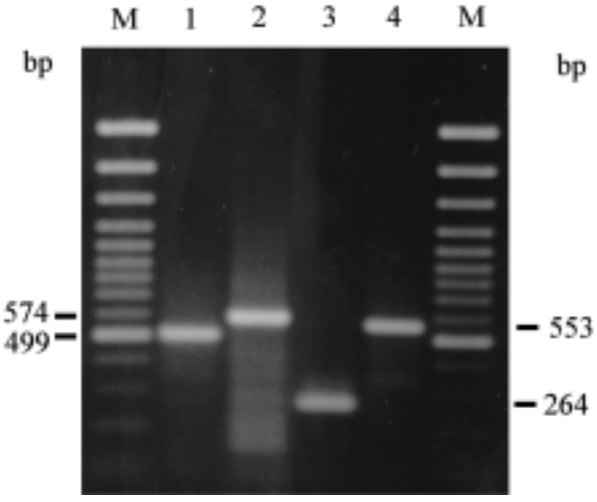
ASGV: 35 cycles of 94°C for 30 s (denaturation) then 55°C for 45 s (for primer pair ASGV-U/ASGV-2) or 62°C for 1 min (for primer pair ASGV-4F/ASGV-4R) (annealing) and 72°C for 2 min (polymerisation), and after the last cycle a final 10 min elongation step at 72°C was added.

PCR products were visualised by electrophoresis in 1.5% agarose gel stained with 0.5 µg/ml of ethidium bromide.

RESULTS AND DISCUSSION

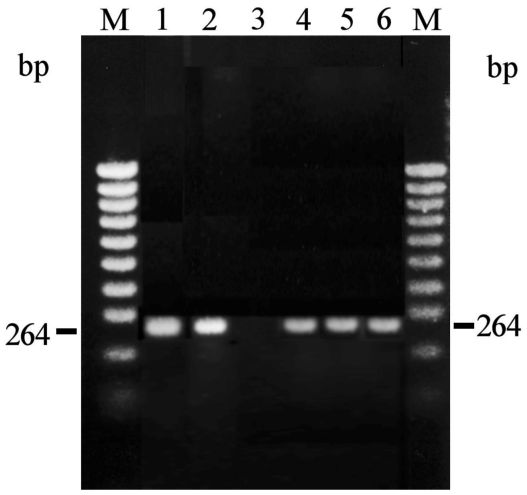
The RT-PCR as presented in this paper was found to be a highly specific and sensitive assay for the detection of the *Apple stem pitting virus* (ASPV) and *Apple stem grooving virus* (ASGV) in both apple and indicator hosts. The selected primers are specific for the detection of both viruses. The specific signal of RT-PCR for ASPV detection is a 264 bp fragment (for primer pair ASP-A/ASP-C) and a 553 bp fragment (for primer pair ASP-I/ASP-II); for ASGV detection this is a 599 bp fragment (for primer ASGV-U/ASGV-2) and a 574 bp fragment (primer pair ASGV-4F/ASGV-4R) as shown in Fig. 1. Although with all four pairs of primers specific amplification products were observed by RT-PCR, the primer pairs ASP-A/ASP-C and ASGV-U/ASGV-2 showed relatively more apple trees with amplification products of RT-PCR of ASPV and ASGV, respectively (KUNDU, unpubl.). Therefore, the primer pairs ASP-A/ASP-C and ASGV-U/ASGV-2 were used for all subsequent testing.

The detection of ASPV and ASGV in apple cultivars is shown in Figs. 2 and 3, respectively. The specific fragment of 264 bp of the ASPV genome (Fig. 2) and that of 499 bp of the ASGV genome (Fig. 3) were amplified with all tested cultivars except with the healthy control. Table 2 summarises the rate of the distribution of ASPV and ASGV viruses among the tested trees, and shows that they were detected frequently by RT-PCR. The incidence of ASGV and ASPV in some cultivars was as high as 16 trees (Spartan) and 13 trees (Idared) out of 20 tested trees. A mixed infection with the two viruses was very common and was recorded in all tested cultivars. As many as 9 out of 20 tested trees of the cultivar Spartan were found to carry mixed infection (Table 2). Mixed infection by three main pome fruit viruses including ASPV, ASGV and *Apple cholorotic leaf spot virus* (ACLSV) has previously been reported (LEONE *et al*. 1998). The present results indicate a high incidence of both viruses in the selected orchard. The presence of ASGV had previously been proved in this orchard by ELISA test (POLÁK & ZIEGLEROVÁ 2001). ELISA-based detection of ASGV has shown over 50% infected trees in tested apple orchards in the Czech Republic (JANEČKOVÁ & PLUHAŘ



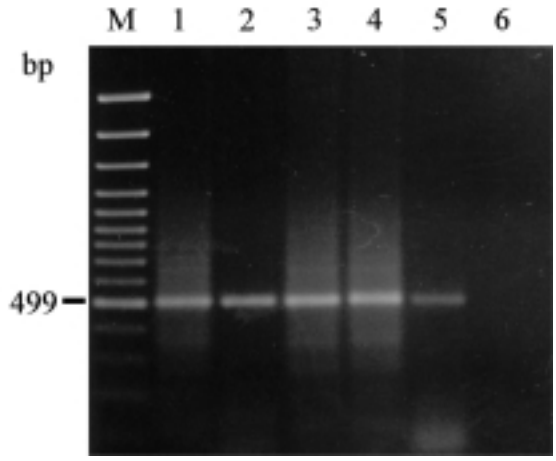
Lane M – molecular marker 100 bp (MBI Fermentas), Lane 1 – primer pair ASGV-2/ASGV-U with specific fragment 499 bp, Lane 2 – primer pair ASGV-4F/ASGV-4R with specific fragment 574 bp, Lane 3 – primer pair ASP-C/ASP-A with specific fragment 264, Lane 4 – primer pair ASP-I/ASP-II with specific fragment 553 bp

Fig. 1. Agarose gel electrophoresis of RT-PCR products of ASPV and ASGV with four pairs of primers



Lane M – molecular marker 100 bp (MBI Fermentas), Lane 1 – ASPV infected *Nicotiana occidentalis* 37B (positive control), Lane 2 – apple cultivar Idared, Lane 3 – healthy control, Lane 4 – apple cultivar Spartan, Lane 5 – apple cultivar Stark Earliest, Lane 6 – apple cultivar Vista Bella

Fig. 2. Agarose gel electrophoresis of RT-PCR products of ASPV with specific fragments 264 bp



Lane M – molecular marker 100 bp (MBI Fermentas), Lane 1 – apple cultivar Idared, Lane 2 – apple cultivar Spartan, Lane 3 – apple cultivar Stark Earliest, Lane 4 – apple cultivar Vista Bella, Lane 5 – ASGV infected *C. quinoa* (positive control), Lane 6 – healthy control

Fig. 3. Agarose gel electrophoresis of RT-PCR products of ASGV with specific fragments 499 bp

1987; POLÁK & ZIEGLEROVÁ 2001). The present results indicate that the use of a sensitive assay like RT-PCR for the detection of the viruses may have shown an even higher level of ASGV incidence. ASPV was also detected at a high frequency by RT-PCR in the tested cultivars of this orchard. However, a final conclusion on the incidence of these viruses can only be made after detailed screening of a larger numbers of orchards. Earlier studies reported the incidence of both viruses in apple nurseries (KUNDU 2001; POLÁK & ZIEGLEROVÁ 2001) and suggested the necessity to evaluate the health condition of the nursery material. Our results correspond to the incidence of ASPV and ASGV reported from Europe (SCHWARZ & JELKMANN 1998; KUMMERT *et al.* 1998) and those of North America (KINARD *et al*. 1996, MACKENZIE *et al*. 1997). The RT-PCR assay offers a very effective and reliable detection method for both ASPV and ASGV viruses. Hence, this assay may be proposed as a standard detection method for the certification system of nursery material in the Czech Republic.

Table 2. The incidence of ASPV and ASGV in tested apple cultivars

Apple cultivars	Number of plants			
	tested	ASPV positive	ASGV positive	with mixed infection of ASPV/ASGV
Idared	20	13	11	6
Spartan	20	11	16	9
Vista Bella	20	9	5	3
Stark Earliest	20	6	15	4

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Souhrn

KUNDU J.K. (2002): **Využití RT-PCR metod pro detekci viru vrásčitosti kmene jabloně a viru žlábkovitosti kmene jabloně u čtyř odrůd jabloní**. Plant Protect. Sci., **38**: 13–17.

Metoda RT-PCR (reverse transcription polymerase chain reaction) byla úspěšně používána pro detekci viru vrásčitosti kmene jabloně (*Apple stem pitting virus* – ASPV) a viru žlábkovitosti kmene jabloně (*Apple stem grooving virus* – ASGV) u čtyř odrůd jabloní (sad starý 25 let). V jednotlivých testovaných odrůdách jabloní byl detekován vysoký podíl infekčních stromů – až 16 stromů pozitivních na ASGV (u odrůdy Spartan) a až 13 stromů pozitivních na ASPV (u odrůdy Idared) mezi 20 testovanými stromy. Byla rovněž zjištěna směsná infekce těchto dvou virů u všech testovaných odrůd (až 9 stromů u odrůdy Spartan).

Klíčová slova: RT-PCR; ASPV; ASGV; virus vrásčitosti kmene jabloně; virus žlábkovitosti kmene jabloně

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