Acta virologica **57**: 435 – 441, 2013 doi:10.4149/av_2013_04_435

Multiplex RT-PCR detection and distribution of four apple viruses in China

Z. JI[1,2], X. ZHAO[1], H. DUAN[1], T. HU[1], S. WANG[1], Y. WANG[1*], K. CAO[1*]

[1]College of Plant Protection, Agricultural University of Hebei, No.2596, South Lucky Street, Baoding, 071001, P. R. China; [2]Research Institute of Pomology, Chinese Academy of Agricultural Sciences, No.98, Chan Bei Street, Xingcheng, 125199, P. R. China

Received March 6, 2013; accepted October 23, 2013

Summary. – Apple trees are natural hosts of four economically important virus species in China. We used a simple, sensitive multiplex RT-PCR protocol with an internal control to simultaneously detect and differentiate four apple viruses: apple chlorotic leaf spot virus (ACLSV), apple mosaic virus (ApMV), apple stem pitting virus (ASPV), and apple stem grooving virus (ASGV). This multiplex RT-PCR could be used as an alternative to other routinely used detection methods. We used this protocol to evaluate the occurrence and distribution of the four apple viruses in China. The four viruses were widely distributed throughout the main apple production region of China, including Heilongjiang, Liaoning, Hebei, Beijing, Henan, Shanxi, Shaanxi, Shandong, Gansu, Ningxia, Xinjiang, Sichuan, and Yunnan. The four viruses, ApMV, ASPV, ASGV, and ACLSV, were present in 80.1%, 65.1%, 73.7%, and 69.7% of the samples, respectively. Two or more of the viruses were detected in most of the infections. The most frequent virus combinations were ApMV + ASGV + ASPV + ACLSV with an incidence of 27.22%, followed by ApMV + ASGV + ACLSV (14.37%), ApMV + ASGV + ASPV (12.54%), and ASGV + ApMV (11.01%). The incidence of the ASPV + ACLSV combination was the lowest (0.61%). This is the first extensive survey conducted in China for monitoring the four apple viruses, which provides important information for apple virus distribution and management in China.

Keywords: apple chlorotic leaf spot virus; apple mosaic virus; apple stem pitting virus; apple stem grooving virus; multiplex RT-PCR; distribution

Introduction

Apple (*Malus x domestica* Borkh.) is one of the most important and widely grown fruit trees in China. The four viruses, apple chlorotic leaf spot virus (ACLSV), apple mosaic virus (ApMV), apple stem pitting virus (ASPV), and apple stem grooving virus (ASGV), occur frequently in mixed infections and cause significant yield reduction in commercial cultivars (Schmidt, 1972; Welsh and Van der Meer, 1989; Zahn, 1996; Desvignes, 1999). It is necessary to use virus-free plant material to reduce the cost of production and prevent virus spread in woody crops. Therefore, certification schemes were established to identify healthy sources for propagation in many countries (Massart *et al.*, 2008). The certification program for virus-free planting material has not been developed in China. Consequently, the risk that viruses spread in orchards through infected planting material is very high. Previous studies on occurrence of apple viruses in China were carried out in 1980s for the three latent viruses and in 1950s for ApMV, which were based only on biological indexing and visual observations (Liu, 1984; Liu and Wang, 1989). During the last decade many new cultivars were introduced and new commercial orchards have been widely planted in China. There is no accurate data on the occurrence of viruses in apple orchards. The aim of this research was to determine the occurrence of the four main viruses, ApMV, ASPV, ASGV, and ACLSV, in apple orchards in China.

Diseases caused by the three latent viruses, ASPV, ASGV, and ACLSV, are usually symptomless (Nemeth, 1986) and ApMV was also reported to have no apparent symptoms in

*Corresponding authors. E-mail: wyn3115347@yahoo.com.cn; ckq@hebau.edu.cn; phone: +86-312-7528157.

Abbreviations: ACLSV = apple chlorotic leaf spot virus; ApMV = apple mosaic virus; ASGV = apple stem grooving virus; ASPV = apple stem pitting virus; DAS-ELISA = double-antibody sandwich ELISA

some apple cultivars (Svoboda and Polák, 2010), therefore, an accurate and sensitive method is needed to detect these viral infections. For routine diagnosis, fast and inexpensive are essential. Therefore, multiplex PCR protocols, which can detect the major virus pathogens affecting one crop simultaneously, provide an alternative (Nie and Singh, 2000; Saade *et al.*, 2000; Sharman *et al.*, 2000; Ito *et al.*, 2002; Gorsane *et al.*, 2005; Wang *et al.*, 2008). At present, the multiplex RT-PCR or multiplex ELISA PCR assays for detection of two or more viruses on apple mainly include: Menzel *et al.* (2002) developed multiplex RT-PCRs that could simultaneously detect virus combinations of ACLSV and ASGV or ASPV and ApMV. Honglyeol *et al.* (2006) created a multiplex RT-PCR assay for the detection of ASGV and ACLSV in infected Korean apple cultivars. Hassan *et al.* (2006) have set up a one-tube pentaplex RT-PCR to simultaneously detect the four apple viruses. Fan *et al.* (2009) established a multiplex RT-PCR assay for simultaneous detection of three latent apple viruses, ASPV, ASGV, and ACLSV.

In the present study we developed a multiplex RT-PCR assay for the detection of ACLSV, ApMV, ASPV, and ASGV plus host plant mRNA in the different apple cultivars in different regions of China using redesigned or published primer sets. All four viruses can be detected from one extract by the multiplex RT-PCR assay with higher sensibility than by DAS-ELISA. In addition, an extensive survey was conducted in China for monitoring the four apple viruses using the multiplex RT-PCR assay.

Materials and Methods

Viruses and plant materials. The positive materials, micropropagated plantlets infected with the four viruses were pretested by DAS-ELISA. To evaluate the distribution of the four apple viruses in China, 327 one-year old branch samples (40 different cultivars of apple) were collected randomly from apple orchards in 13 provinces in China, including Heilongjiang, Liaoning, Hebei, Beijing, Henan, Shanxi, Shaanxi, Shandong, Gansu, Ningxia, Xinjiang, Sichuan and Yunnan. All samples were stored at -80°C until nucleic acid extraction. Healthy samples pretested by DAS-ELISA were used as the negative control.

Extraction of total nucleic acids. Total nucleic acids were extracted with the method described originally by Menzel *et al.* (2002). Bark chips containing the vegetative buds from the apical 0-5cm of one-year old branch were used for extraction.

Virus-specific primers. Redesigned and published primer sets were used in this study. The redesigned primers were based on the conserved region of the available genomes from the GenBank. The alignments were carried out with DNAMAN software package program (Lynnon BioSoft, Quebec City, Canada). The primer sequences, primer positions and expected amplified fragment size for each template are given in Table 1.

Multiplex RT-PCR assay. cDNA synthesis of 4-viruses infected tissue culture plantlets and the 327 field samples used for amplification were primed with hexanucleotides (random primers, Sangon, Shanghai, China) according to the M-MLV reverse-transcriptase manufacturer (Takara), in a final volume of 20 µl. Healthy apple tissue was included in each round. Multiplex RT-PCR was performed using the cDNA from either the tissue culture plantlets or field samples as a template, and with primers for the four viruses and *EF1-α*. Amplifications were carried out in a 25 µl reaction mixture. 12.5 µl 2×Taq MasterMix (NEB), 4 µl cDNA products and 4.6 µl Nuclease-free water were used for DNA amplification. The reaction was performed with the mixture of the all virus primer pairs at a final concentration of 0.48 µmol/l for ASGV and ACLSV, 0.16 µmol/l for ASPV, 0.4 µmol/l for ApMV. *EF1*-α primers were used as an internal control (*EF1-α*-F, -R, each at 0.15 µmol/l). The PCR program was performed in a standard thermocycler (Bioer, Shanghai, China), with the following conditions: denaturation at 94°C for 2 min; followed by 35 cycles of

Table 1. Properties of multiplex RT-PCR primers for four viruses pathogenic on apples in China

Primer name	Sequence 5′–3′	Primer position[a]	Product size	Reference
ASPV-F	TGGAACCTCATGCTGCA	8878-8894	360 bp	Redesigned
ASPV-R	TTGGGATCAACTTTACTAAAAAGCATAA	9211-9238		
ASGV-F	GAGGATTTAGGTCCCTCTC	5595-5613	821 bp	Redesigned
ASGV-R	GTATAAAGGCAGGCATGTCAACC	6394-6416		
ApMV-F	CAACCGAGAGGTTGGCA	1585-1600	161bp	Candresse *et al.* (1998)
ApMV-R	TTCTAGCAGGTCTTCATCGA	1769-1788		
ACLSV-F	GAGAGTTTCAGTTTGCTAGACA	6745-6766	566 bp	Park *et al.* (2006)
ACLSV-R	GCAAATTCAGTCTGTAAAAG	7291-7310		
EF1-α[b]-F	ACCAACCTTGACTGGTACAAGG	710-731	236 bp	Park *et al.* (2006)
EF1-α-R	TGGTGCATCTCAACAGACT	926- 945		

Footnote: F = sense primer; R = antisense primer; a = the reference Acc. Nos. (National Center for Biotechnology Information) for the determination of the primer positions are D14996 for apple chlorotic leaf spot virus, D21829 for apple stem pitting virus, U15608 for apple mosaic virus, D14995 for apple stem grooving virus and AJ223969 for the EF1-α (elongation factor 1α) gene; b = internal control.

denaturizing at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min; and a final extension at 72°C for 7 min. The PCR products (8 µl) were analyzed by electrophoresis in 2.0% agarose gel containing 0.5 µg ml^{-1} ethidium bromide in 1× TAE buffer (40 mmol/l tris-acetate, 1 mmol/l EDTA) for 40 min at 120 V. Reactions with and without a target template were included in every experiment as a blank and positive control, respectively.

Cloning and sequencing. In order to confirm the identity of the amplified products, fragments of each virus and the internal control were purified using the DNA gel extraction kit (TaKaRa) and cloned into the pGEM-T vector (Promega). The recombinant vectors were transformed into *Escherichia coli* strain JM109. The PCR-derived clones with an insert of the expected size were selected for sequencing using the dideoxynucleotide chain termination method using an automated sequencer (Perkin-Elmer Applied Biosystems). The sequences obtained were compared with the corresponding virus retrieved from the GenBank database.

DAS-ELISA assay. A commercially available DAS ELISA kit (Bioreba AG, Reinach, Switzerland), which can differentiate the four viruses with great sensitivity and specificity, was used according to the manufacturer′s instructions.

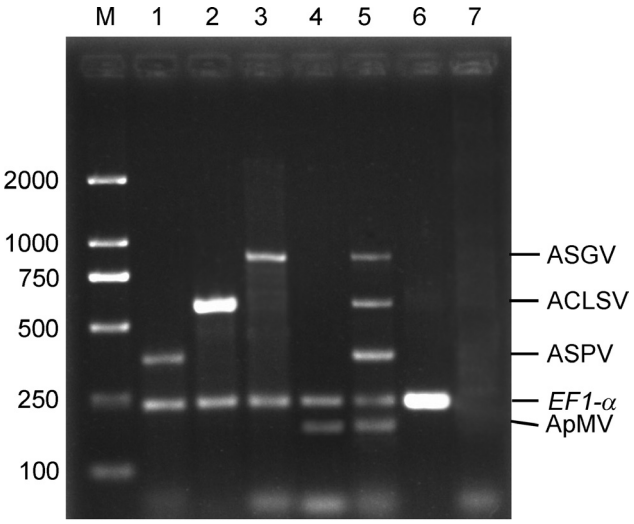

Fig. 1

Analysis of mixed virus extracts by the multiplex RT-PCR and uniplex RT-PCR

DNA size marker DL2000 (M), ASPV primers in uniplex RT-PCR (1), ACLSV primers in uniplex RT-PCR (2), ASGV primers in uniplex RT-PCR (3), ApMV primers in uniplex RT-PCR (4), primers for ASGV, ACLSV, ASPV, and ApMV in uniplex RT-PCR (5), healthy control (6), water control (7).

Results

Amplification of DNA from infected tissue culture plantlets by the multiplex RT-PCR

The tissue culture plantlets infected with ASGV, ACLSV, ASPV, and ApMV were used to optimize the multiplex RT-PCR detection assay. The primer mixture of the four viruses and *EF1-α* for simultaneous amplification of the four viruses and the internal control were tested. The uniplex PCR reaction using each virus and the *EF1-α* primer pairs produced five specific products: ASGV (821 bp), ACLSV (566 bp), ASPV (360 bp), ApMV (161 bp), and *EF1-α* (236 bp) (Fig.1, lane 1–4). For the multiplex PCR (Fig.1, lane 5), all primers were mixed together in a single reaction. This produced the same four virus products and the internal control, indicating the high specificity of the designed primers and the absence of any interaction or interference among them. No differences were observed between the uniplex RT-PCR for each virus using only the specific primer pair and multiplex RT-PCR procedure using a mixture of all primers in the same reaction, demonstrating that interference problems did not occur.

In order to confirm the specificity of the assay, the PCR products were purified and ligated into the pGEM-T-easy vector (Promega) and recombinant plasmids from transformed cells were subjected to sequence analysis. In all cases, the DNA sequences were obtained as expected for each virus (data not shown).

Detection limits of the multiplex RT-PCR assay

Total nucleic acids extracted from the infected tissue culture plantlets with the four viruses were serially diluted in total nucleic acid extracts from healthy control plants and used for multiplex RT-PCR and uniplex RT-PCR. We detected specific bands for ASGV and ACLSV at dilutions of up to 1:500, ASPV at dilutions up to 1:1000, and ApMV at dilutions of up to 1:100 in the multiplex RT-PCR (Fig. 2a). As expected, a specific RT-PCR product for the internal control could be detected at all dilutions in the multiplex assay without any obvious difference in band intensity (Fig. 2a). The expected PCR fragments for the four viruses were observed in the uniplex system till the dilutions of 1:500, 1:500, 1:2,000, and 1:200 of the original total nucleic acid extracts for ASGV, ACLSV, ASPV, and ApMV, respectively (Fig. 2b). The multiplex RT-PCR sensitivity was similar to the uniplex RT-PCR for all four viruses.

Incidence of the four apple viruses in apple producing areas in China detected by the multiplex RT-PCR assay

In order to examine the occurrence and distribution of apple viruses in China, 327 samples were collected randomly from the major apple-producing areas in 13 provinces in China. The samples included 40 different commercial cultivars. All these samples were tested by the multiplex RT-PCR

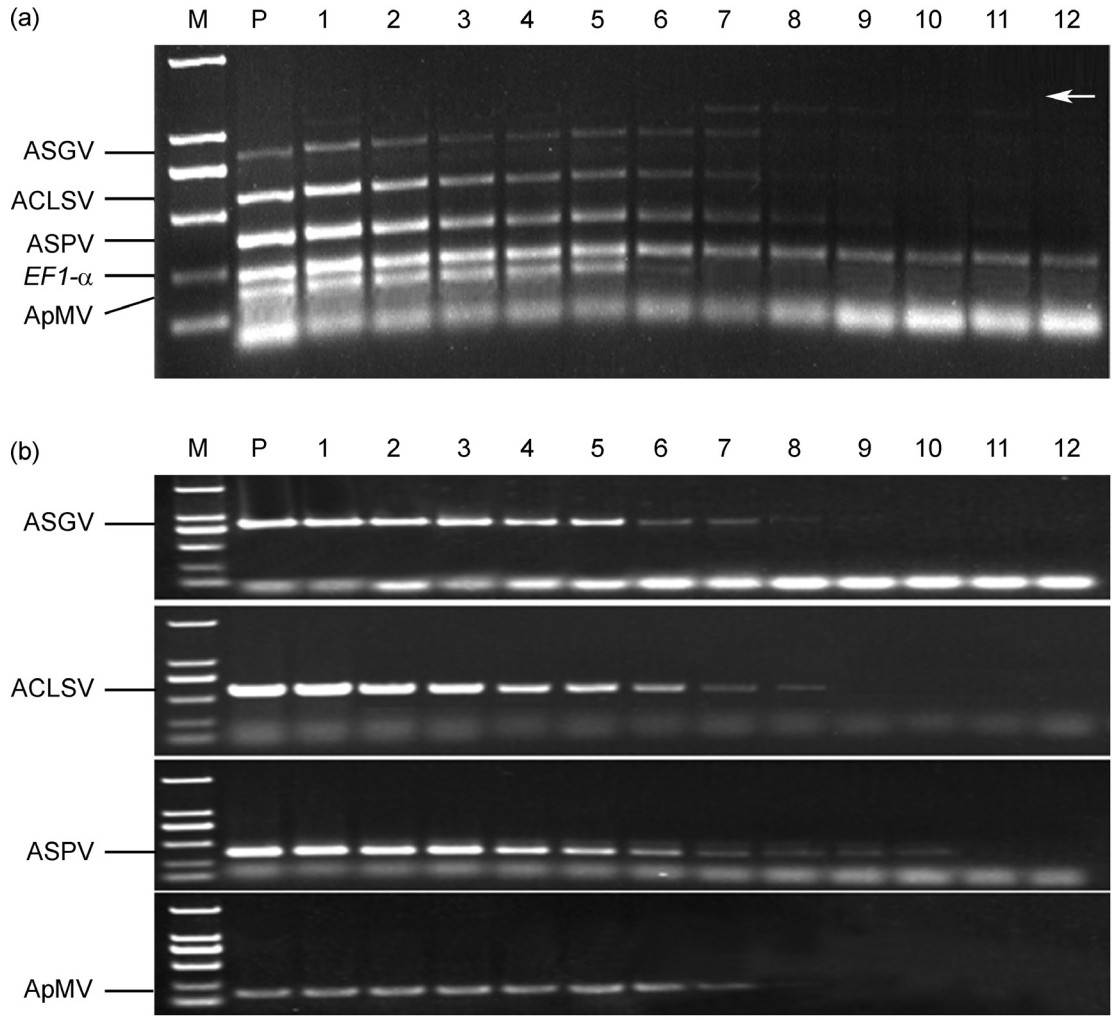

Fig. 2

Comparison of the sensitivities of the multiplex RT-PCR and uniplex RT-PCR for the detection of ASGV, ACLSV, ASPV, and ApMV

Agarose gel electrophoresis analysis of amplification products of multiplex RT-PCR (a) and uniplex RT-PCR (b), obtained from total nucleic acid extract of a four virus-infected sample serially diluted in total nucleic acid extract of a healthy control plant (lanes 1–12 from left to right refer to dilutions 1:2, 1:5, 1:10, 1:20, 1:40, 1:100, 1:200, 1:500, 1:1000, 1:2,000, 1:5,000, 1:10,000), undiluted total nucleic acid extract (P), DNA size marker DL2000 (M), The additional fragment in infected samples is indicated by an arrow.

assay. The results (Table 2) showed that all of the field samples tested (327 in total) were positive for at least one of the four apple viruses and the four viruses were detected in almost all 13 surveyed provinces, including Heilongjiang, Liaoning, Hebei, Beijing, Henan, Shanxi, Shaanxi, Shandong, Gansu, Ningxia, Xinjiang, Sichuan, and Yunnan. ASPV was not found only in Sichuan. The average positive rates of ApMV, ASPV, ASGV, and ACLSV were 80.1%, 65.1%, 73.7%, and 69.7% respectively. The phenomenon of complex infection was very predominant (Table 3). 75.23% of the infected apple plants harbored more than one species of virus. The most frequent virus combinations were ApMV + ASGV + ASPV + ACLSV with an incidence of 27.22%, followed by ApMV + ASGV + ACLSV (14.37%), ApMV + ASGV + ASPV (12.54%), and ASGV + ApMV (11.01%), respectively. The incidence of ASPV + ACLSV combination was the lowest (0.61%). Fig. 3 shows the results obtained from ten representative infected apple samples in the multiplex RT-PCR assay, comprising the samples 15-7, 7-5, 7-6, 11-5, 13-1, 2-2, 15-9, 17-1, 9-10, 6-5. Four virus bands of different sizes were observed only in infected samples but not in negative controls.

In order to test whether the multiplex RT-PCR assay is suitable and reliable for diagnosis of ASGV, ACLSV, ASPV, and ApMV in field samples, 40 samples selected randomly

Table 2. Multiplex RT-PCR assays of 327 samples of bark tissue from 40 different apple cultivars and 13 provinces in China

Collection areas	No. of sites	No. of samples/cultivars	Positive rate (%)			
			ApMV	ASPV	ASGV	ACLSV
Heilongjiang	1	10/Jinhong, Longfeng, Longguan	90	100	90	100
Liaoning	3	20/Jonathan, Wangjianghong, Sansa, Golden Delicious, Liangxiang, Alps Otome, Gaolonghanfu, J ieke11, Fuji, Orin	85	100	90	50
Hebei	15	109/Fuji,Starking,Ralls Janet, Shengli, Golden Delicious, Sansa, Xinshiji, Danxia, Shinsckai, Jonagold, Red Delicious	71.5	75.2	75.2	67.9
Beijing	3	10/Fuji	100	80	90	60
Henan	8	30/Bene Shogun, Red Fuji, k12, Balenghaitang, Gala, Yuhuazaofu, Starking	83.3	70	66.7	86.7
Shanxi	2	10/Fuji	90	90	70	50
Shaanxi	7	44/Qinguan, Fuji, Qianqiu, Gala, Red Fuji, Xinnonghong, Lifu, Micui	72.7	52.3	81.8	65.9
Shandong	5	20/Bene Shogun, Nanfangcui, Yoko, Honglu, Weixi, Hongtailang	80	75	50	65
Gansu	2	20/Starkrimson, Red Fuji	85	75	40	60
Ningxia	1	10/Matsumoto Nishiki, Fuji, Ningguan, Gala, Golden Delicious	100	10	100	70
Xinjiang	1	20/Golden Delicious, Fuji, Gala	75	25	95	70
Sichuan	5	10/Fuji	100	0	70	70
Yunnan	3	14/Red Fuji, Golden Delicious	100	78.6	100	57.1
All samples	56	327	80.1	65.1	73.7	69.7

from 327 field samples were analyzed by ELISA using virus-specific antibodies (Bioreba, Switzerland). All tested samples matched between ELISA and RT-PCR results except for four samples, which were positive for ASGV in multiplex RT-PCR, while negative in ELISA.

Discussion

The production of virus-free planting material is the key for effective control of apple virus, because of no natural vector is reported for spreading the four viruses (Ŝutic, *et al.*., 1999). Thus a sensitive, reliable and rapid method for virus detection is needed. In the present study, a multiplex RT-PCR assay was developed for simultaneous detection of ACLSV, ASGV, ASPV, and ApMV on apple, which is simple procedure able to identify single or mixed virus infections. Most of the RT-PCR assays from previous studies cannot cover the four viruses in one assay except for the method developed by Hassan *et al.* (2006). For some ApMV isolates from China, no product could be amplified using Hassan′s method in our previous work. This is likely the result of two nucleotides mutation corresponding to the upstream primer of ApMV occurring in the genome of these isolates (data not shown). The method developed by us proved to be sensitive, reliable and rapid in detecting the four viruses from most common apple cultivars and different apple producing regions in China. The detection limit was similar to the uniplex RT-PCR for all four viruses. An additional faint fragment of about 1500 bp could be observed on the gel using RNA template at the dilutions from 1:200 to 1: 5,000, indicated by an arrow in Fig. 2a, but the detection accuracy was not decreased. In order to validate the method, 40 field samples randomly selected from 327 samples were tested by commercially available DAS-ELISA kit for the presence of the four viruses. The results showed that multiplex RT-PCR was more sensitive than ELISA for ASGV, since more infected samples were detected by multiplex RT-PCR than by ELISA, which is in accordance with previous reports (Menzel *et al.*, 2003; Hassan *et al.*, 2006). The multiplex RT-PCR assay would be valuable in testing large numbers of field samples or tissue culture plantlets in diagnostic clinic and research setting. Early detection is very important for the control of apple virus disease, because there are no effective methods for disease management. The assay plays an important role in predicting virus infection at its initial stage and alert

Table 3. Mixed infections of four viruses in apple orchards in China

Virus type	No. positive	Positive rate (%)
ApMV+ASPV	10	3.06
ApMV+ASGV	36	11.01
ApMV+ACLSV	22	6.73
ASPV+ASGV	13	3.98
ASPV+ACLSV	2	0.61
ACLSV+ASGV	25	7.65
ApMV+ASPV+ASGV	41	12.54
ApMV+ASPV+ACLSV	28	8.56
ApMV+ASGV+ACLSV	47	14.37
ASPV+ASGV+ACLSV	22	6.73
ApMV+ASPV+ASGV+ACLSV	89	27.22
All	246	75.23

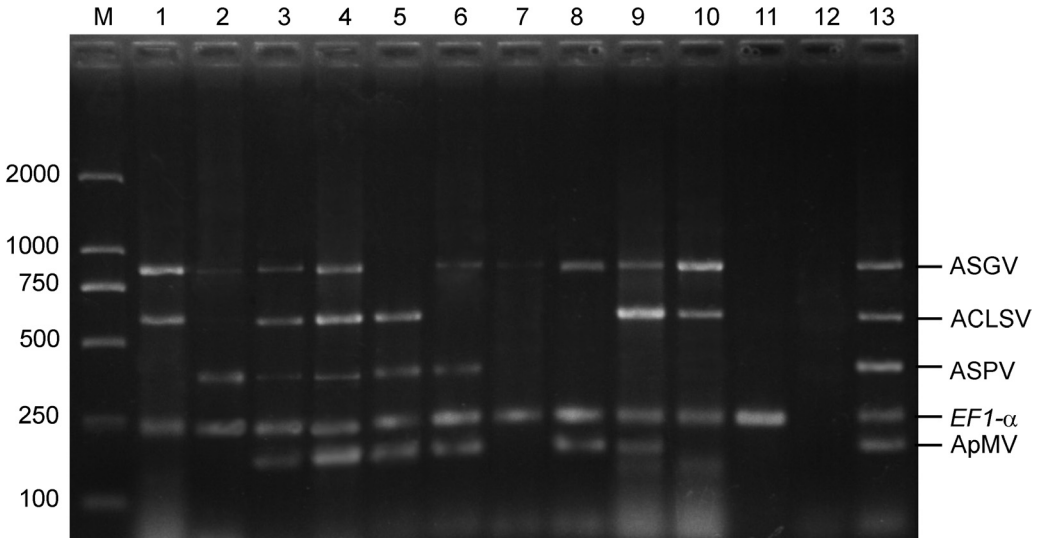

Fig. 3

Representative results for the detection of ASGV, ACLSV, ASPV, and ApMV by multiplex RT-PCR

DNA size marker DL2000 (M), RT-PCR products of the infected field samples, 15-7, 7-5, 7-6, 11-5, 13-1, 2-2, 15-9, 17-1, 9-10, and 6-5 (1–10), RT-PCR product of healthy control (11), RT-PCR product of water control (12), RT-PCR product of positive control (13).

maintenance personnel before severe damage of apple trees in the field occurs.

We collected 327 samples from the major apple-producing areas in China, including 13 provinces and 40 different commercial cultivars, which represented a wide geographical distribution and host diversity. The samples were tested for the four viruses by the multiplex RT-PCR assay. The results showed that ApMV, ASPV, ASGV, and ACLSV were the predominant virus species in the apple fields of China and were found in the majority of commonly grown cultivars and in almost all thirteen surveyed provinces in China. There is no significant correlation between the incidence rate of the viruses and climate of different planting regions. It is not clarified whether there is a correlation between the incidence rate and apple cultivars because of the limited sample number. As described by Posnette *et al.* (1963), field-grown apple trees often harbored complex mixtures of several different virus species and our results confirmed the phenomenon. Most of the infected apple plants (75.23%) harbored more than one virus species, most often four viruses, and a few plants were infected with two or three viruses. To our knowledge, this is the first systematical analysis of the infection with ACLSV, ASGV, ASPV, and ApMV on apples in China. High infection rates of these four viruses in varietal collections revealed that they might spread through infected budwoods to commercial apple orchards quickly and widely.

Because a certification scheme for planting material is not established in China, there is a very high risk for continuous spread and further evolution of aggressive isolates in the orchards. The data indicates a great need to establish a certification scheme for fruit tree propagation in China and other countries, where it is not yet established.

Acknowledgements. We thank Prof. John Hu and Dr. Fred Brooks for critical review and discussion of the manuscript, Prof. Jianshe Sun for providing the positive material with the four apple viruses, the 25 Apple Experimental Stations for collecting apple field samples. Financial support was provided by the research funds from National Research Center for Apple Industry (nycytx-08-04-01), National Agricultural Scientific Fund for Publicbenefit (200903004-42), National Nature Science Foundation (31201487), Hebei Natural Science Foundation (C2013204058) and Hebei Natural Science Foundation of Higher Education Institutions for Youth (2011245).

References

Candresse T, Koflvi SA, Lanneav M, Dunez J (1998): A PCR-ELISA Procedure for the simultaneous detection and identification of prunus necrotic ringspot (PNRSV) and apple mosaic (ApMV) ilarviruses. Acta Hortic. 472, 219–225.

Desvignes JC (1999): Virus diseases of fruit trees. Ctifl, Paris. EPPO, Certification schemes-Pathogen-tested material of Malus, Pyrus and Cydonia. EPPO Bull, 29, 239–252.

Fan X, Dong Y, Zhang Z, Li L, Pei G (2009): Multiplex RT-PCR assay for simultaneous detection of three latent apple viruses. Acta Hortic Sin. 36, 1821–1826.

Gorsane F, Gharsallah-Chouchene S, Nakhla MK, Fekih-Hassan I, Maxwell DP, Marrakchi M, Fakhfakh H (2005): Si-

multaneous and rapid differentiation of members of the tomato yellow leaf curl viruscomplex by multiplex PCR. J. Phytopathol. 87, 43–48.

Hassan M, Myrta A, Polak J (2006): Simultaneous detection and identification of four pome fruit viruses by one-tube pentaplex RT-PCR. J. Virol. Methods 133, 124–129. http://dx.doi.org/10.1016/j.jviromet.2005.11.002

Ito T, Ieki H, Ozaki K (2002): Simultaneous detection of six citrus viroids and Apple stem grooving virus from citrus plants by multiplex reverse transcription polymerase chain reaction. J. Virol. Methods 106, 235–239. http://dx.doi.org/10.1016/S0166-0934(02)00147-7

Liu F, Wang H (1989): Study on latent viruses of apple. II. identification of latent viruses in apple cultivars and dwarf rootstocks. Acta Phytopathol. Sin. 19, 193–197.

Liu F (1984): Occurrence and research status of apple Virus in China. Plant Quarantine 5, 6–10.

Massart S, Brostaux Y, Barbarossa L, Cesar V, Cieslinska M, Dutrecq O, Fonseca F, Guillem R, Lavina A, Olmos A, Steyer S, Wetzel T, Kummert J, Jijakli MH (2008): Inter-laboratory evaluation of a duplex RT-PCR method using crude extracts for the simultaneous detection of Prune dwarf virus and Prunus necrotic ringspot virus. European J. Phytopathol. 122, 539–547.

Menzel W, Jelkmann W, Maiss, E (2002): Detection of four apple viruses by multiplex RT-PCR assays with coamplification of plant mRNA as internal control. J. Virol. Methods 99, 81–92. http://dx.doi.org/10.1016/S0166-0934(01)00381-0

Menzel W, Zahn V, Maiss E (2003): Multiplex RT-PCR-ELISA compared with bioassay for the detection of four apple viruses. J. Virol. Methods 110, 153–157. http://dx.doi.org/10.1016/S0166-0934(03)00112-5

Nemeth M (1986): Viruses, Mycoplasma and Ricketsia Diseases of Fruit Trees. (Boston: Kluwer Academic Publishers).

Nie X, Singh RP (2000): Detection of multiple potato viruses using an oligo (dT) as a common cDNA primer in multiplex RT-PCR. J. Virol. Methods 86, 179–185. http://dx.doi.org/10.1016/S0166-0934(00)00140-3

Park H, Yoon J, Kim H, Baek K (2006): Multiplex RT-PCR Assay for the detection of ASGV and ACLSV in infected Korean apple cultivars. Plant Pathol. J. 22, 168–173. http://dx.doi.org/10.5423/PPJ.2006.22.2.168

Posnette AF, Cropley R, Ellenberger CE (1963): The effect of virus infection on the growth and crop of apple, pear, and plum trees. Phytopathol. Mediterr. 2, 158–161.

Saade M, Aparicio F, Sanchez-Navarro JA, Herranz MC, Myrta A, Di Terlizzi B, Pallaz V (2000): Simultaneous detection of three ilarviruses affecting stone fruit trees by nonisotopic molecular hybridization and multiplex reverse transcription polymerase chain reaction. Phytopathology 90, 1330–1336. http://dx.doi.org/10.1094/PHYTO.2000.90.12.1330

Schmidt H (1972): The effect of 'latent' virus infections on the yield of maiden trees on 20 apomictic apple seedling rootstocks. J. Hortic. Sci. Biotech. 47, 159–163.

Sharman M, Thomas JE, Dietzgen RG (2000): Development of a multiplex immunocapture PCR with colourimetric detection for viruses of banana. J. Virol. Methods 89, 75–88. http://dx.doi.org/10.1016/S0166-0934(00)00204-4

Ŝutic DD, Ford ER, Tos˘ic MT (1999): Virus diseases of fruit trees. In Sulzycki J (Ed.): Handbook of Plant Virus Diseases. Boca Raton, FL, USA, CRC Press LLC, pp. 321–389.

Svoboda J, Polák J (2010): Relative concentration of Apple mosaic virus coat protein in different parts of apple tree. Hort. Science 37, 22–26.

Welsh MF, van der Meer FA (1989): Apple stem grooving. In Fridlund PR (Ed.): Virus and Virus-like Diseases of Pome Fruits and Simulating Noninfectious Disorders. Pullman: Cooperative Extension College of Agriculture and Home Economics, Washington State University, pp. 127–137.

Wang X, Zhou C, Tang K, Zhou Y, Li Z (2008): A rapid one-step multiplex RT-PCR assay for the simultaneous detection of five citrus viroids in China. Eur. J. Plant Pathol. 124, 175–180. http://dx.doi.org/10.1007/s10658-008-9386-y

Zahn V (1996): Obstvirustestung im Wandel der Zeit. Obstbau. 21, 547–550.