KJOAS

ISSN (Print) : 2466-2402
ISSN (Online) : 2466-2410

PLANT & FOREST

Occurrence of *Apple stem grooving virus* in commercial apple seedlings and analysis of its coat protein sequence

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Abstract

Apple stem grooving virus (ASGV), *Apple chlorotic leaf spot virus* (ACLSV), and *Apple stem pitting virus* (ASPV) have been known to induce top working disease causing economical damage in apple. Occurrences of these three viruses in pome fruit trees, including apple, have been reported around the world. The transmission of the three viruses was reported by grafting, and there was no report of transmission through mechanical contact, insect vector, or seed except some herbaceous hosts of ASGV. As RNA extraction methods for fruit trees, Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) and multiplex RT-PCR techniques have been improved for reliability and stability, and low titer viruses that could not be detected in the past have become detectable. We studied the seed transmission ability of three apple viruses through apple seedling diagnosis using RT-PCR. Nineteen seeds obtained from commercially grown apple were germinated and two of the resulting plants were ASGV positive. Seven clones of the amplified ASGV coat protein (CP) genes of these isolates were sequenced. Overall sequence identities were 99.84% (nucleotide) and 99.76% (amino acid). Presence of a previously unreported single nucleotide and amino acid variation conserved in all of these clones suggests a possible association with seed transmission of these 'S' isolates. A phylogenetic tree constructed using ASGV CP nucleotide sequences showed that isolate S sequences were grouped with Korean, Chinese, Indian isolates from apple and Indian isolates from kiwi.

Keywords: apple stem grooving virus, seed transmission, RT-PCR

Citation: Han JY, Park CH, Seo EY, Kim JK, JohnHammond, Lim HS. 2016. Occurrence of *Apple stem grooving virus* in commercial apple seedlings and analysis of its coat protein sequence. Korean Journal of Agricultural Science 43:21-27.

DOI: http://dx.doi.org/10.7744/kjoas.20160003

Editor: Kee Woong Park, Chungnam National University, Korea

Received: December 1, 2015

Revised: February 28, 2016

Accepted: March 2, 2016

Introduction

Apple stem grooving virus (ASGV), *Apple chlorotic leaf spot virus* (ACLSV), and *Apple stem pitting virus* (ASPV) are major viruses of apple which lead to economicdamage and top working disease (Campbell, 1963; Posnette et al., 1963; Schmidt, 1972; Yanase, 1983; Zahn, 1996; Wang et al., 2011). All three viruses belong to genera within the Betaflexiviridae,

with ASGV belonging to genus *Capillovirus*, ACLSV to the genome *Trichovirus,* and ASPV to the genome *Foveavirus*.

These three viruses are transmitted by grafting in the field or mechanically to diagnostic species (e.g. *Chenopodium quinoa* or *Chenopodium amaranticolor* etc.) (Yanase, 1983; Llacer et al., 1985; Kinard and Scott, 1996). ASGV (under the synonym *Citrus tatter leaf virus*) has been reported to be transmitted through seed of lily and *C. quinoa* (Inouye et al., 1979), and at a low rate in seed of citrus (Tanner et al., 2011). Viruses or viroids that are able to transmit through apple seed include *Apple latent spherical virus* (ALSV), *Tomato bushy stunt virus* (TBSV), *Tobacco mosaic virus* (TMV), *Apple scar skin viroid* (ASSVd), and *Dapple apple viroid* (DAV) (Allen, 1969; Hadidi et al., 1991; Nakamura et al., 2011). However, there were no reports of seed transmission in apple of any of the three viruses examined here.

We tested apple seedlings to check the possibility of seed transmission of the above three viruses. Apple seedlings were germinated from seeds of three commercially sold apple cultivars, and RT-PCR was used for virus detection. ASGV was detected in two seedlings but ACLSV and ASPV were not detected.

The ASGV genome is composed of a positive sense single stranded RNA with a length about 6,495-6,497 nucleotides (Yoshikawa et al., 1992). Open reading frame 1 (ORF1) encodes the replication-associated protein (Rep) and coat protein (CP) as a continuous polyprotein separated by a variable region. Rep contains several conserved domains (methyltransferase, papain-like protease, RNA helicase, and RNA-dependent RNA polymerase) and is translated directly from genomic RNA. Although the 27 kDa CP sequence is present in the ORF1 polyprotein, CP is expressed from a 3'-co-terminal subgenomic RNA (Magome et al., 1997; Tatineni et al., 2009). ORF2 encodes the 37 kDa movement protein (MP) which is located between Rep and CP in a different reading frame, overlapping the variable region of ORF1.

To carry out further analysis of the detected ASGV, seven coat protein clones (of isolate S) were isolated and sequenced. There was a conserved nucleotide at CP position 299 and amino acid at CP residue 100, which differ from other reported ASGV CP sequences. Phylogenetic tree analysis suggests that the detected ASGV-S CP is grouped with those of other Korean ASGV isolates reported in 2015 (Han et al., 2015).

Materials and Methods

Apple seeds

All apple seeds were obtained from commercially sold apple fruits during September to December in 2014. Cultivars were Busa, Hongok, and Aori, and mixed randomly. Obtained seeds were washed and kept at 4℃ until used. In order to break seed dormancy, seeds were soaked in water and kept at 4℃ for 4-7 days. Subsequently, seeds were placed on wet tissue in petri dishes covered with aluminum foil and kept at 24℃ until seeds germinated. Germinated seeds were planted in soil and kept at 24℃ under light for 16 hours and darkness for 8 hours.

Diagnosis

Apple leaves were frozen in liquid nitrogen and ground with beads. 50-100 mg of ground tissue were mixed with 450 µl RLT buffer from the RNeasy Plant Mini Kit (Qiagen) containing 4.5 µl of 2-mercaptoethanol. Total RNA extraction steps were performed according to the manufacturer's protocol. Total RNAs were then reverse transcribed into cDNA using SuPrimeScript RT Premix (2x) (GENET BIO CO., Ltd) as recommended

by the manufacturer's protocol. PCR was performed in a 20 μl total volume containing 1 μl cDNA, 10 pmol of virus-specific primers (Table 1), 2 μl of 10X Reaction Buffer, 2 μl of 10 mM dNTPs mixture, and 1 unit of Prime Taq DNA polymerase (GENET BIO CO., Ltd). The PCR conditions were as follows: 5 min at 94 °C for pre-denaturing, 37 cycles of 30 sec at 94 °C for denaturing, 30 sec at 56 °C for annealing, and 30 sec at 72 °C for extension, followed by 5 minutes at 72 °C for final extension. Plants were recorded as positive if PCR products of the expected sizes (Table 1) were obtained, and no products were obtained from negative controls.

Table 1. Primers used in this study.

Name	Sequence (5'-3')	Feature	Expected size
For *Apple stem grooving virus* (ASGV) diagnosis[z]			
ASGV_F_484bp	CATCTGATAAGACCCAGTTTCC	-	488bp
ASGV_R_484bp	TTACTCTCCGAACCTGCCTC		
For *Apple chlorotic leaf spot virus* (ACLSV) diagnosis			
ACLSV_F	TTCATGGAAAGACAGGGGCAA	Menzel et al., 2002	677bp
ACLSV_R	AAGTCTACAGGCTATTTATTATAAGTCTAA		
For *Apple stem pitting virus* (ASPV) diagnosis			
ASPV_F	ATGTCTGGAACCTCATGCTGCAA	Menzel et al., 2002	370bp
ASPV_R	TTGGGATCAACTTTACTAAAAAGCATAA		
For ASGV coat protein amplification[z]			
ASGV_CP_F	AAAGTCGACATGAGTTTGGAAGACGTGCTTC	*Sal* Ⅰ	714bp
ASGV_CP_R	AAAGGATCCCTAACCCTCCAGTTCCAGGTTAC	*BamH* Ⅰ	

[z]Primers designed based on alignment of multiple ASGV sequences (Han et al., 2015)

Cloning and Sequencing

The ASGV CP gene was amplified from cDNAs of ASGV positive samples, with PCR performed as described above with ASGV CP specific primers (Han et al., 2015; Table 1), except that 30 cycles were carried out, with a 1-minute extension time. The PCR products were cloned into pGEM®-T Easy vector (Promega CO., LTD.). Plasmids with PCR product inserts were confirmed using restriction enzyme digestion with *Sal*I and *BamH*I (New England BioLabs® Inc.). Positive plasmids were sequenced by Macrogen Inc.

Sequence analysis and phylogenetic tree construction

The nucleotide sequences and amino acid sequences were compared using DNAMAN software (Version 5. 2.10, Lynnon BioSoft). Phylogenetic trees were constructed using neighbor-joining method with 1,000 bootstrap replicates in the MEGA version 6 (Tamura *et al.*, 2013). 48 ASGV CP nucleotide sequences were obtained from NCBI GenBank. Cherry virus A (CVA; *genus*: *Capillovirus*) CP nucleotide sequence was used as an outgroup to root the ASGV CP phylogenetic tree.

Results and Discussion

Diagnosis of apple viruses

Seeds of three commercially obtained apple cultivars (Busa, Hongok, and Aori) were germinated and tested to detect apple viruses. Total RNAs from 3-6 true leaves were subjected to RT-PCR using three sets of virus-specific primers for ASGV, ACLSV, and ASPV (Table 1). Two of nineteen seedling plants were positive for ASGV. Neither ACLSV or ASPV were detected in any plants (Table 2).

Table 2. Accession numbers, isolate names, host, and geographic origin of ASGV sequences obtained from National Center for Biotechnology Information (NCBI) and used for coat protein sequence comparison and phylogenetic tree analysis.

Accession number	Name	Host	Country
KR606307	GW2	Apple	Korea
KR606308	GW3	Apple	Korea
KR606310	YS1	Apple	Korea
KR606316	CS6	Apple	Korea
KR606322	CJ17	Apple	Korea
KR606323	CW6	Apple	Korea
KR606324	CW8	Apple	Korea
JN871585	LJ-1	Apple	China
JN871590	ZT-2	Apple	China
JN871586	ML-1	Apple	China
KF735124	YT-4-4	Apple	China
JX885571	HY	Apple	China
JX885575	LX	Apple	China
JQ308181	ASGV-CHN	Apple	China
JX885570	HL	Apple	China
JN871587	TJX-1	Apple	China
KP025666	WZMG-1	Mandarin	China
AY886760	KRL-1	Pear	China
FJ608985	P-4-1-69	Pear	China
GQ330293	P-6-1-17	Pear	China
JX080201	AC	Apple	Germany
LN627005	Ap-VD	Apple	India
HG796198	Ki-3	Kiwi	India
HG796197	Ki-2	Kiwi	India
LN559086	WR-1	Rose	India
AB004063	Li-23	Lily	Japan
D14455	CTLV	Lily	Japan
JN792476	A67	Apple	Korea
JN792477	A50	Apple	Korea
JN792473	A135	Apple	Korea
JN792490	P161	Pear	Korea
JN792494	P12	Pear	Korea
FJ355920	LCd-NA-1	Orange	Taiwan
EU553489	CTLV-ML	Lemon	USA

Comparison of ASGV CP sequences

Complete ASGV CP genes were cloned and sequenced for analysis of sequence variability. A total of seven ASGV CP sequences were determined from the seedlings (isolate S: S8, S20, S22, S27, S31, S33, and S38,

respectively). All of them showed slightly different nucleotide sequences. However, S20, S22, and S31 had an identical sequence at the amino acid level. Overall sequence identities were 99.84% at the nucleotide level and 99.76% at the amino acid level.

ASGV CP isolate S sequences were compared with reported ASGV CP sequences from multiple countries and hosts (Table 2). Nucleotide and amino acid sequence results showed that a single amino acid sequence variation was observed only in the seven isolate S clones. All isolate S clones showed thymine at nt 299, whereas previously reported ASGV CP sequences showed cytosine at that position (Fig. 1). Amino acid sequence corresponding to nt 299 showed Isoleucine for all clones of isolate S, compared to Threonine for all previously reported ASGV CP sequences.

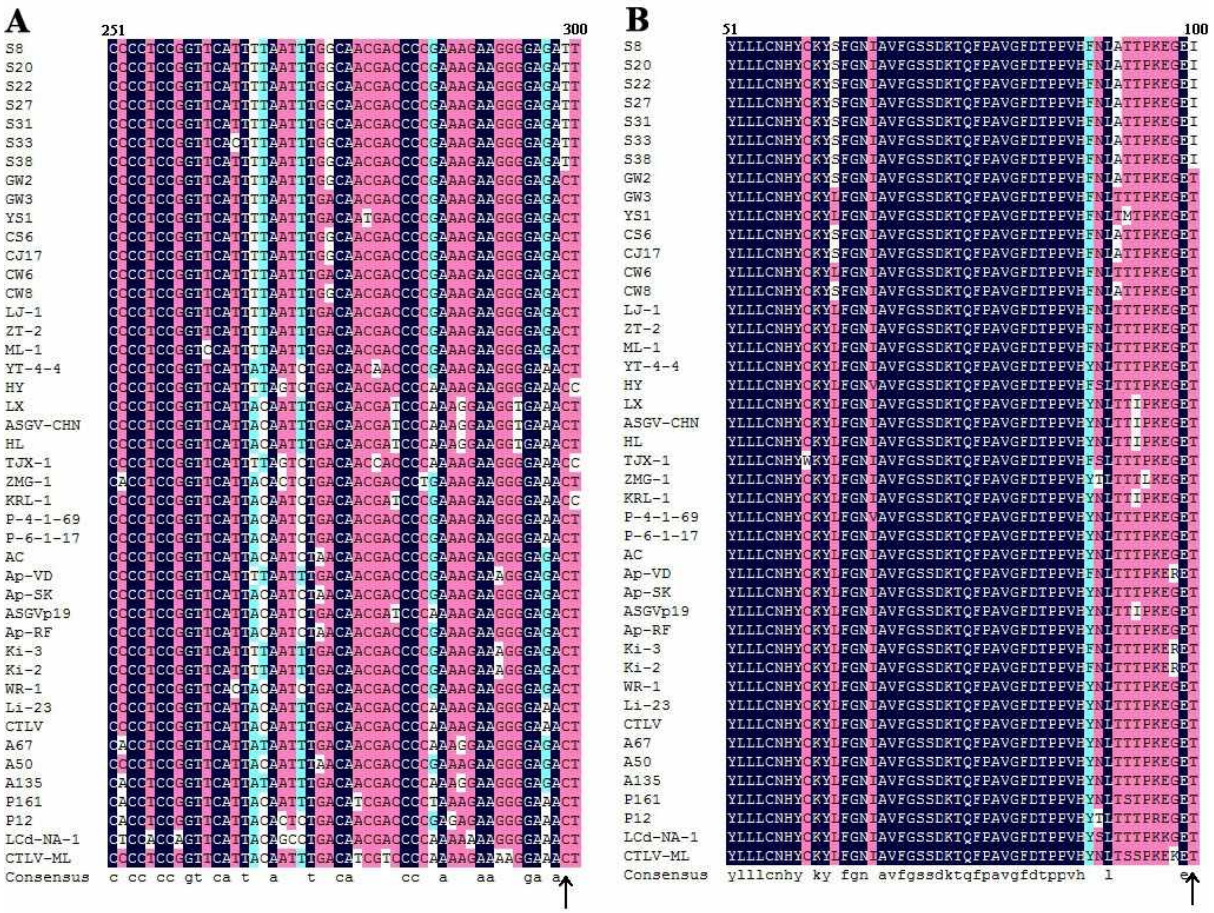

Fig. 1. ASGV CP nucleotide alignment result (A) and amino acid alignment result (B). Sequence alignment was performed with DNAMAN 6.0 program. The characters shaded in navy blue are consensus sequences and in light blue, white, pink are nucleotide (A) or amino acid (B) substitutions. Arrows indicate single nucleotide variation (A, isolate S: thymine, the other clones: cytosine) and single amino acid variation (B, isolate S: Iso-leucine, the other clones : Threonine) observed only in all clones of isolate S.

Phylogenetic tree analysis

An ASGV CP phylogenetic tree was constructed using nucleotide sequences of seven isolate S clones with other sequences from multiple countries and hosts (Fig. 2). All isolate S clones were grouped with seven Korean isolates, three Chinese isolates from apple, two isolates from kiwi, and an Indian isolate from apple.

Average nucleotide sequence identity among all isolates used for constructing the phylogenetic tree was 95.92%. Average nucleotide identity of the group of all isolate S sequences and their most closely related isolates was 99.81%.

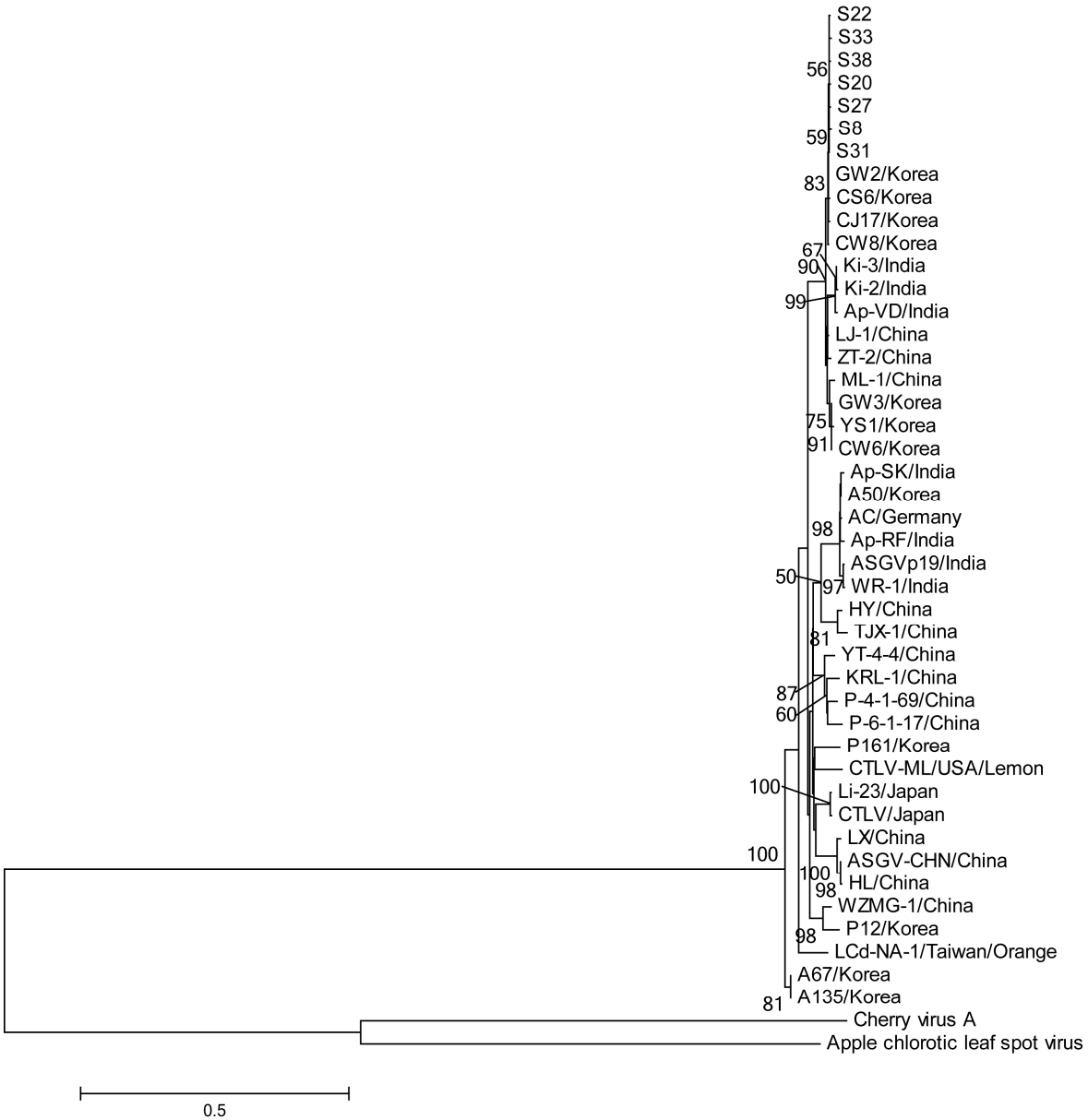

Fig. 2. Phylogenetic tree based on ASGV coat proteins. The tree was constructed with MEGA 6.0 using neighbor-joining method with 1,000 bootstrap replicates; 37 isolates of ASGV CP, one of *Cherry virus A* and *Apple chlorotic leaf spot virus* CP for analysis were obtained from National Center for Biotechnology Information GenBank. The name indicates the name and country of isolates. The numbers at the nodes are bootstrap values above 50%. The scale bar indicates the number of nucleotide substitutions.

Acknowledgements

This study was financially supported by research fund of Chungnam National University in 2014.

References

Allen WR. 1969. Occurrence and seed transmission of *Tomato bushy stunt virus* in apple. Canadian Journal of Plant Science 49:797-799.

Campbell AI. 1963. The effect of some latent virus infections on the growth and cropping of apples. Journal of Horticultural Science 38:15-19.

Hadidi A, Hansen AJ, Parish CL, Yang X. 1991. Scar skin and dapple apple viroids are seed-borne and persistent in infected apple trees. Research in Virology 142(4):289-296.

Han JY, Kim JK, Cheong JS, Seo EY, Park CH, Ju HK, Cho IS, Gotoh T., Moon JS, Hammond J, Lim HS. 2015. Survey of *Apple chlorotic leaf spot virus* and *Apple stem grooving virus* occurrence in Korea and frequency of mixed infections in Apple. Journal of the Faculty of Agriculture, Kyushu University. 60(2):323-329.

Inouye N, Maeda T, Mitsuhata K. 1979. *Citrus tatter leaf virus* isolated from lily. Annals of the Phytopathological Society of Japan 45(5):712-720.

Kinard G, Scott S. 1996. Detection of Apple chlorotic leaf spot and *Apple stem grooving virus*es using RT-PCR. Plant Disease 80(6):616-621

Llacer G, Cambra M, Lavina A, Aramburu J. 1985. Suitable conditions for detecting *apple chlorotic leaf spot virus* in apricot trees by enzyme-linked immunosorbent assay (ELISA). Agronomie 5(9):809-812.

Magome H, Terauchi H, Yoshikawa N, Takahashi T. 1997. Analysis of double-stranded RNA in tissues infected with *Apple stem grooving capillovirus*. Annals of the Phytopathological Society of Japan 63:450-454.

Nakamura K, Yamagishi N, Isogai M, Komori S, Ito S, Yoshikawa N. 2011. Seed and pollen transmission of *Apple latent spherical virus* in apple. Journal of General Plant Pathology 77:48-53.

Posnette AF, Cropley R, Ellemberger C. 1963. The effect of virus infection on the growth and crop of apple, pear and plum trees. Phytopathologia Mediterranea 2:158-161.

Schmidt H. 1972. The effect of 'latent' virus infections on the yield of maiden trees on 20 apomictic apple seedling rootstocks. Journal of Horticultural Science 47(2):159-163.

Tanner JD, Kunta M, Da Graça JV, Skaria M, Nelson SD. 2011. Evidence of a low rate of seed transmission of *Citrus tatter leaf virus* in citrus. Phytopathology 101:S175.

Tatineni S. Afunian MR, Gowda S, Hilf ME, Bar-Joseph M, Dawson W. 2009. Characterization of the 5'- and 3'-terminal subgenomic RNAs produced by a *Capillovirus*: Evidence for a CP subgenomic RNA. Virology 385(2):521-528.

Wang L, Hong N, Matić S, Myrta A, Song Y, Michelutti R, Wang G. 2011. Pome fruit viruses at the Canadian Clonal Genebank and molecular characterization of *Apple chlorotic leaf spot virus* isolates. Scientia Horticulturae 130(3):665-671.

Yanase H. 1983. Back transmission of *Apple stem grooving virus* to apple seedlings and induction of symptoms of apple topworking disease in Mitsuba kaido (*Malus sieboldii*) and Kobano zumi (*Malus sieboldii* var. arborescens) rootstocks. Acta Horticulturae 130:117-122.

Yoshikawa N, Sasaki E, Kato M, Takahashi T. 1992. The nucleotide sequence of A*pple stem grooving capillovirus* genome. Virology 191(1):98-105.

Zahn V. 1996. Obstvirustestung im Wandel der Zeit. Obstbau 21:547-550.