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Survey of *Apple Chlorotic Leaf Spot Virus* and *Apple Stem Grooving Virus* Occurrence in Korea and Frequency of Mixed Infections in Apple

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Due to the absence of knowledge of the distribution of *Apple stem grooving virus* (ASGV) and *Apple chlorotic leaf spot virus* (ACLSV) in apples in Korea, we carried out a survey for these viruses in Gyeongsang and Chungcheong provinces in 2014. A total of 65 samples were collected and tested by RT–PCR using ASGV and ACLSV specific primers. ASGV was detected in 22 samples, and ACLSV in three samples; two of the samples showed double infection of ASGV and ACLSV. Phylogenetic analysis suggests that Korean ASGV and ACLSV were introduced from other countries. Prevalence of ASGV and ACLSV indicates that virus prevention and control may be poorly managed in orchards. Since fruit trees remain in the orchard for many years and it is not possible to eliminate virus from infected trees, healthy scions and virus–resistant rootstocks must be used for virus control. Because it is difficult to visually distinguish ASGV–infected and ACLSV–infected apple trees from healthy trees, thorough surveys by molecular biology methods must be performed routinely.

Key words: *Apple stem grooving virus*, *Apple chlorotic leaf spot virus*, double infection, RT–PCR

INTRODUCTION

Apple (*Malus domestica*) belongs to the family *Rosaceae* and is cultured worldwide. About 10 cultivars are cultured in Korea. Outdoor fruit tree cultivation area in Korea was 153,415 ha, and the apple tree cultivation area was 30,702 ha, which is the largest component of the fruit cultivation area (Statistics Korea, 2014). The main production areas of apple in Korea are Gyeongsang Province and Chungcheong Province which account for 92% of the domestic apple output (Statistics Korea, 2014).

Apple stem grooving virus (ASGV) and *Apple chlorotic leaf spot virus* (ACLSV) are major viruses causing economic damage to apples (Campbell, 1963; Posnette *et al*., 1963; Zahn, 1996) and significant annual losses have been reported. ASGV is the type species of the genus *Capillovirus*. The genome is positive sense single stranded RNA with a length of about 6,495–6,497 nucleotides (Yoshikawa *et al*., 1992) excluding the poly(A) tail. The viral RNA has 2 ORFs; ORF1 encodes a Replication–associated protein and a coat protein (CP) (27 kDa) which is expressed from subgenomic RNA (Tatineni *et al*., 2009). ORF2 encodes a movement protein (36kDa) in a different reading frame from ORF1. ACLSV is the type species of the genus *Trichovirus*, and has a positive sense single strand RNA genome of about 7,474–7,555 nucleotides excluding the poly(A) tail (German *et al,* 1990; Sato *et al*., 1993). The viral RNA has 3 ORFs; ORF1 encodes a replication–associated protein, ORF2 encodes a movement protein (MP)(50 kDa), and ORF3 encodes a coat protein (21 kDa).

These two viruses are the major pathogens of apple which induce topworking disease (Schmidt, 1972; Wang *et al*., 2011). Typically, visible symptoms do not appear except in some cultivars (Nemeth, 1986). Both viruses can concurrently infect one host, causing greater damage to fruit production than either single virus infection (Campbell, 1963; Cembali *et al*., 2003). The worldwide occurrence of ASGV and ACLSV has been reported, especially in China (Pūpola *et al*., 2011; Song *et al*., 2011; Wang *et al*., 2011; Kumar *et al*., 2012; Ji *et al*., 2013; Duan *et al*., 2014; Liu *et al*., 2013, 2014). In Korea, the occurrence has also been reported (Park *et al*., 2006; Cho *et al*., 2010; Kim *et al*., 2011). The distribution of ASGV and ACLSV has not been studied in Korea after 2010. There was a previous report of nucleotide sequence of Korean ASGV isolates (Shim *et al*., 2004) but not of ACLSV isolates. In order to investigate management condition of apple ochards after 2010, our research team performed virus diagnosis for apple samples collected from Gyeongsang Province and Chungcheong Province in 2014. Also, we identified complete nucleotide sequences of Korean ASGV CP and ACLSV MP in order to phylogenetically compare Korean isolates to those from other countries. These results provide data on the prevalence

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and potential introduction route of these viruses, and should aid establishment of prevention measures, as well as providing primary data for characterization of these apple viruses.

MATERIALS AND METHODS

SAMPLE COLLECTION

Collection of samples was performed from May to November of 2014 (Figure 1). ASGV and ACLSV do not induce characteristic symptoms. Thus, leaves and fruits of apple trees showing reduced production, declining health, poor coloring, malformation and maldevelopment, which are considered normal virus effects, were selected (Figure 2). A total of 65 samples were collected from Gyeongsang Province and Chungcheong Province apple orchards.

DIAGNOSIS

Total RNAs of collected samples were extracted by a minor revision of the Cetyltrimethylammonium bromide (CTAB) isolation method (Chang *et al*., 1993) and cDNAs were synthesized using RevertAid Reverse Transcriptase (Thermo Fisher Scientific Inc.). Primers for ASGV diagnosis were designed based on ASGV coat protein nucleotide sequence (Accession number: D14995) from National Center for Biotechnology Information (NCBI), and primers for ACLSV diagnosis were those reported by Menzel *et al*. (2002) (Table 1). RT–PCR was performed in a 20 μl total volume containing 1 μl cDNA, 10 pmol of each primers, 2 μl of 10X Reaction Buffer, 2 μl of 10 mM dNTPs mixture and 1 unit of Prime Taq (GENET BIO CO., Ltd) or Blend Taq® (Toyobo CO., Ltd). The PCR conditions were as follows: 5 minutes at 94°C for pre–denaturing, 35 cycles of 30 seconds at 94°C for denaturing 30 seconds at 56°C for annealing, and 30 seconds at 68°C for extension, followed by 5 minutes at 68°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.

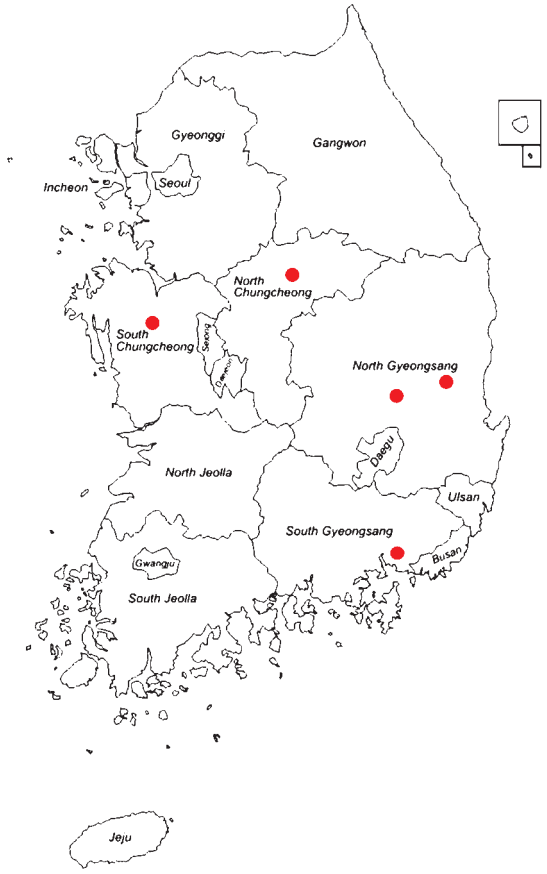

Fig. 1. Geographical distribution of apple sample collection sites.

POLYMERASE CHAIN REACTION, CLONING, SEQUENCING

The ASGV CP gene and ACLSV MP gene were amplified from all of virus positive samples using above cDNAs and appropriate virus–specific primers. PCR was performed as described above, except that 30 cycles were carried out, with a 1 minute extension time for ASGV CP, and 1 minute 30 seconds for ACLSV MP amplification. The PCR products were visualized in 1% agarose gel stained with ethidium bromide under UV light. The PCR products were cloned into pGEM®–T Easy vector

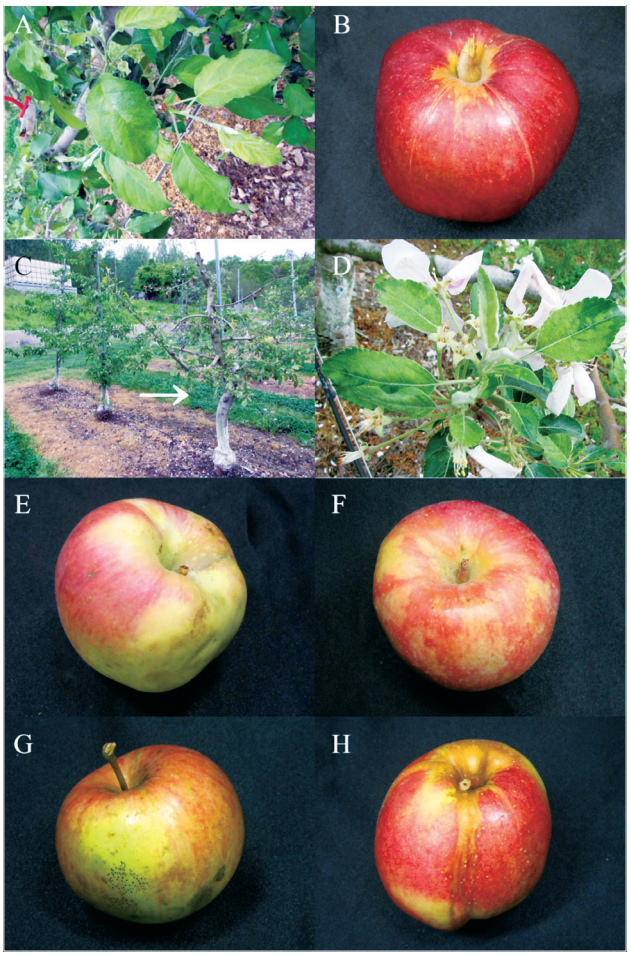

Fig. 2. Various symptoms of collected samples. *Apple chlorotic leaf spot virus* (ACLSV) and *Apple stem grooving virus* (ASGV) Double infected leaves (A) and fruit (B); ACLSV infected tree showing shorter height than surrounding trees and pale green leaf color (white arrow)(C); ASGV infected leaves (D) and fruits (E, F, G and H).

Table 1. Primers used in this study

Virus	Name	Sequence (5' – >3')	Feature	Expected size
ASGV	For ASGV[a] diagnosis			
	ASGV_F (5'oligo)	CATCTGATAAGACCCAGTTTCC	Menzel *et al* (2002)	273bp
	ASGV_R (3'oligo)	TTACTCTCCGAACCTGCCTC		
	ASGV_F_484bp (5'oligo)	CATCTGATAAGACCCAGTTTCC		484bp
	ASGV_R_484bp (5'oligo)	TTACTCTCCGAACCTGCCTC		
	For amplifying complete ASGV CP			
	ASGV_CP_F (5'oligo)	AAA GTCGAC ATGAGTTTGGAAGACGTGCTTC	***Sal*I**	714bp
	ASGV_CP_R(3'oligo)	AAA GGATCC CTAACCCTCCAGTTCCAGGTTAC	***BamH*I**	
ACLSV	For ACLSV[b] diagnosis			
	ACLSV_F (5'oligo)	TTCATGGAAAGACAGGGGCAA	Menzel *et al* (2002)	677bp
	ACLSV_R (3'oligo)	AAGTCTACAGGCTATTTATTATAAGTCTAA		
	For amplifying complete ACLSV MP			
	ACLSV_MP_F(5'oligo)	AAA GTCGAC ATGATGATAAGGGGTCAC	***Sal*I**	1377bp
	ACLSV_MP_R(3'oligo)	AAA GGGCCC TCACACACCTGGCGGAAAG	***Apa*I**	

[a] *Apple stem grooving virus*
[b] *Apple chlorotic leaf spot virus*

(Promega Co., Ltd.) or T–blunt™ vector (Solgent Co., Ltd.). Plasmids with PCR product inserts were confirmed using restriction enzyme digestion with *SalI* and *BamHI* for ASGV CP, or *SalI* and *ApaI* for ACLSV MP (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 (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). 17 ACLSV MP nucleotide sequences and 30 ASGV CP nucleotide sequences were obtained from NCBI GenBank. *Cherry mottle leaf virus* (CMLV; *genus*: *Trichovirus*) MP nucleotide sequence and Cherry virus A (CVA; *genus: Capillovirus*) CP nucleotide sequence were used as the outgroup for ACLSV and ASGV phylogenetic trees respectively.

RESULTS AND DISCUSSION

DISTRIBUTION OF APPLE VIRUSES

In order to investigate the occurrence of apple viruses in Korea, total 65 suspected virus–infected apple samples were subjected to RT–PCR. Among these 65 samples, 22 were positive for ASGV (33.8%), three samples were positive for ACLSV (4.6%) and dual infections of ASGV and ACLSV were diagnosed in two samples (3.1%) (Table 2). ASGV occurrence was confirmed throughout the entire collection region. The ASGV infection ratio was 48% in Gyeongsang Province and 25% in Chungcheong Province. However, ACLSV occurred at only two sites (Gunwi and Chungju) with an infection ratio of 8% in Gyeongsang Province and 2.5% in Chungcheong Province.

In an earlier survey of Korean apples, the infection ratio of ASGV was 35.2%, that of ACLSV was 50.9% and

Table 2. Regional virus diagnosis results

Province	Region	ASGV positive	ACLSV positive	Double infection
Gyeongsang	Gunwi	9/16	2/16	1/16
	Cheongsong	1/7	0/7	0/7
	Changwon	2/2	0/2	0/2
	Total	12/25 (48%) [a]	2/25 (8%)	1/25 (4%)
Chungcheong	Yesan	6/25	0/25	0/25
	Chungju	4/15	1/15	1/15
	Total	10/40(25%)	1/40(2.5%)	1/40(2.5%)
Total		22/65(33.8%)	3/65(4.6%)	2/65(3.1%)

[a] positive samples/samples tested, diagnosis (percentage)

double infection of ASGV and ACLSV was 19.6% (Park *et al*., 2006). In contrast to those results, ACLSV infection ratio was 4.6% in this study. However, the ASGV infection ratio in the current study slightly higher, at 48% in Gyeongsang province. Cho *et al*. (2010) reported that infection rates of ASGV and ACLSV were 74.24% and 0.4% respectively in Korean pears. Although based on relatively small sample sizes, these numbers suggest that infection rates differ between orchards, and between apples and pears, but imply that prevention against viruses has been ineffective or not performed in Korea orchards.

SEQUENCE VARIABILITY OF CP GENE OF ASGV AND MP GENE OF ACLSV

All virus positive samples were subjected to PCR to amplify the complete ASGV CP or ACLSV MP gene. ASGV CP is a conserved region that is under negative selection (Liebenberg *et al*., 2012) and has been used for ASGV phylogenetic analysis (Lui *et al*., 2013; Shim *et al*., 2006; Bhardwaj *et al*., 2014;). ACLSV MP, which is also known to function as a systemic silencing suppressor, has been well studied for its biological features and mechanisms (Yoshikawa *et al*., 1999, 2000, 2006; Satoh *et al*., 2000; Isogai *et al*., 2003; Isogai and Yoshikawa, 2005; Yaegashi *et al*., 2007, 2008).

Eighteen ASGV isolates were obtained from all regions and 6 ACLSV isolates were obtained from Gunwi (Table 3). Although ACLSV was detected from one Chungju sample, we failed to obtain a full–length ACLSV MP product from this sample. This failure may have been due to sequence variability with the 5' or 3' terminal regions targeted by the ACLSV MP primers.

The nucleotide sequences and amino acid sequences of the isolates were compared using DNAMAN (Version 5.2.10). The results indicated that ASGV isolate pairwise identity was an average of 99.16% (nucleotide) and 98.95% (amino acid). ACLSV isolates showed an average of 92.06% (nucleotide) and 95.09% (amino acid) identity.

Phylogenetic trees were constructed based on the CP nucleotide sequences of 18 Korean ASGV isolates from this study and 30 additional ASGV isolates from NCBI, and on the MP nucleotide sequences of 6 Korean ACLSV isolates from this study and 17 other isolates from NCBI GenBank (Figure 3). The GenBank sequences originate from various countries and hosts. ASGV isolates are separated into three groups: ASGV CP Group 1 included 18 isolates from this study, 2 isolates from Indian kiwi and 4 isolates from Chinese apple. Groups 2 and 3 include isolates from multiple countries and hosts. ACLSV MP sequences were separated into 2 groups, of which Group 1 separated into three subgroups. Isolates from this study were all included in Group 1. Subgroup 1A included CLGW1, CLGW2 and 4 isolates from Japanese

Table 3. Regions and accession numbers of isolates of this study (Accessions are being processed)

Virus	Isolate	Region	Accession number
Apple stem grooving virus	GW2	Gunwi	KR606307
	GW3		KR606308
	GW4		KR606309
	YS1	Yesan	KR606310
	YS4		KR606311
	YS5		KR606312
	YS8		KR606313
	YS10		KR606314
	CS3	Cheongsong	KR606315
	CS6		KR606316
	CS7		KR606317
	CS8		KR606318
	CS9		KR606319
	CJ6	Chungju	KR606320
	CJ16		KR606321
	CJ17		KR606322
	CW6	Changwon	KR606323
	CW8		KR606324
Apple chlorotic leaf spot virus[b]	GW1	Gunwi	KR606325
	GW2		KR606326
	GW3		KR606327
	GW4		KR606328
	GW6		KR606329
	GW8		KR606330

and Chinese apple. Subgroup 1B included CLGW3, CLGW6, one isolate from Indian apple, 3 isolates from French, Chinese, and German plum. Subgroup 1C included CLGW4, CLGW8 and 4 isolates from only Japanese apple. Group 2 included 3 isolates from Chinese pear, 1 Chinese hawthorn isolate and 1 Japanese apple isolate.

Two Korean ASGV CP isolates from GenBank (A50: Cheongsong, A67: Chungju) are from the same regions as some isolates of this study (CS3, 6, 7, 8, 9: Cheongsong, C6, 16, 17: Chungju). However, the phylogenetic tree (Figure 3A) separated these isolates into three distinct groups. The collection year of isolates A50 and A67 was 2008 and 2009 respectively. These data suggest that the new ASGV isolates detected in Korea may have been introduced from either China or India. Korean ACLSV

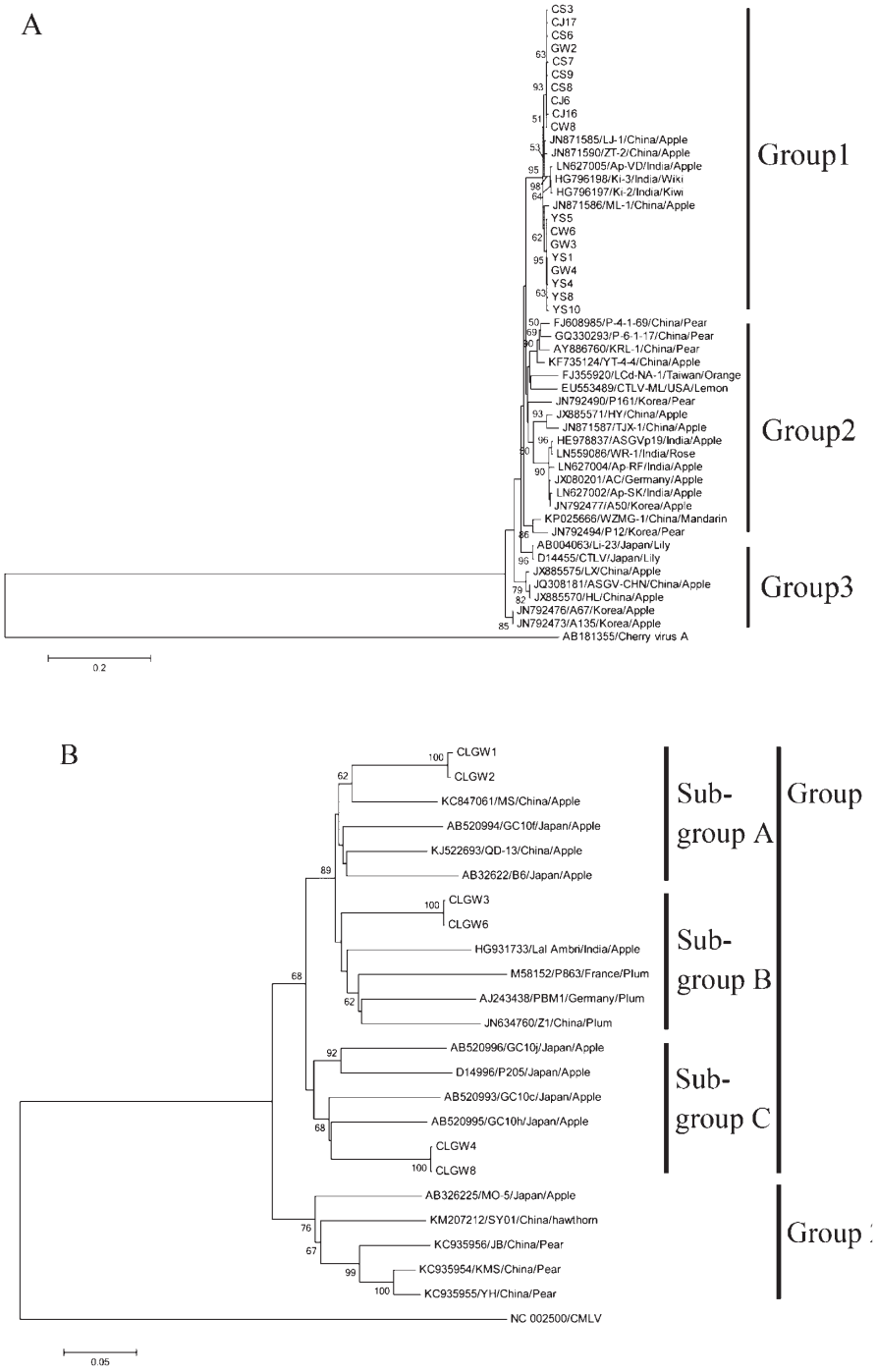

Fig. 3. Phylogenetic tree based on *Apple stem grooving virus* coat proteins(A) and *Apple chlorotic leaf spot virus* movement proteins(B). The trees were constructed with MEGA 6.0 using Neighbor-joining method with 1,000 bootstrap replicates. 30 isolates of ASGV CP and 17 isolates of ACLSV MP for analysis were obtained from National Center for Biotechnology Information GenBank. The name indicates Accession numbers/name of isolate/country/host. The number at the nodes is bootstrap values above 50%. The scale bars indicate the number of nucleotide substitutions. CMLV = *Cherry mottle leaf virus*.

isolates have been poorly investigated to date; there is only one prior nucleotide sequence (Accession number: LC006864) and the corresponding amino acid sequence (BAP81778) of partial ACLSV CP. The current study is the first report of the MP sequences of Korean ACLSV isolates and phylogenetic analysis. The Group 1 of the ACLSV MP tree (Fig. 3 B) is separated into three subgroups. Each subgroup was divided by countries and host of isolates. Considering that the isolates from this study were from single region, Gunwi, and divided between all 3 subgroups, we suggest that there are at least three distinct subgroups of ACLSV isolates in Korea, and that more biological and phylogenetic data are needed to trace the potential origins of ACLSV isolates in Korea.

ASGV prevalence has remained high since 2006. The nucleotide and amino acid identity of the isolates in this study was above 98%, and the phylogenetic tree showed that isolates from all collection sites were clustered in a single group with Chinese and Indian isolates. However, previously reported Korean isolates were phylogenetically distinct from the isolates identified in this study, but group with additional sequences from China and India. This suggests that the ASGV isolates from different orchards came from a limited number of sources, but likely originate from either China or India, and have been distributed within Korea with either budwood (most likely) or rootstocks. In contrast, ACLSV was much less prevalent in the orchards tested, but isolates showed greater sequence diversity (only 92–95% identity between isolates) and were distributed among three subgroups of isolates with multiple countries of origin, This suggests at least three separate introductions into Korea, and the potential for recombination between isolates or transmission to other hosts. ASGV and ACLSV are both transmitted by grafting sensitive rootstock and virus infected scions. Both viruses may also be mechanically transmitted to some diagnostic species (e.g. *Chenopodium quinoa* etc.) (Yanase, 1982; Llacer *et al*., 1985; Kinard and Scott, 1996). Thus, supply and usage of healthy scion and resistant rootstock is important in order to prevent spread of these apple viruses. A national survey of apple viruses should be performed routinely, and additional biological and phylogenetic studies of Korean ASGV and ACLSV isolates should be accomplished. Virus–certified budwood and rootstocks should be produced and made available for establishment of new orchards to allow eventual elimination of infected orchards.

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