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Molecular Characterization and Distribution of *Apple Chlorotic Leaf Spot Virus* on Apple in China

Hao Duan[1], Zhirui Ji[2], Shutong Wang[1], Tongle Hu[1], Yanan Wang[1] and Keqiang Cao[1]

1 College of Plant Protection, Agricultural University of Hebei, No. 2596, South Lucky Street, Baoding 071001, China
2 Research Institute of Pomology, Chinese Academy of Agricultural Sciences, No. 98, Xing Hai Street, Xingcheng 125199, China

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Correspondence
Y. Wang or K. Cao, College of Plant Protection, Agricultural University of Hebei, Baoding, China.
E-mails: wyn3215347@163.com and ckq@hebau.edu.cn

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Abstract

Apple chlorotic leaf spot virus (ACLSV) is one of the most economically important latent viruses infecting apple in China. This is the first report of the almost complete nucleotide sequence and the characterization of the genome of a Chinese isolate (ACLSV-MS, GenBank Accession Number KC847061) from apple. Based on the genome nucleotide sequence, ACLSV-MS showed the highest identity (99.4%) to isolate ACLSV-B6 (GenBank Accession Number AB326224) from apple in Japan and the least identity (69.5%) with isolate TaTao5 (GenBank Accession Number: EU223295) from peach in the USA. The occurrence and distribution of ACLSV in China were also recorded. Three hundred and twenty-seven apple samples (40 different cultivars) collected from 56 sites in 13 provinces of China were tested by RT-PCR. The virus was detected in all regions surveyed (the provinces of Heilongjiang, Liaoning, Hebei, Beijing, Henan, Shanxi, Shaanxi, Shandong, Gansu, Ningxia, Xinjiang, Sichuan and Yunnan), with an average incidence of 69.7%. The positive samples in Heilongjiang province were highest with an incidence of 100% followed by Henan province with an incidence of 86.7%. The positive samples in Liaoning and Shanxi were the lowest with an incidence of 50%. The occurrence of virus in five common cultivars was determined. The percentage of ACLSV was highest in cv. Gala with an incidence of 33.3%, while lowest in cv. Starking with an incidence of 18.2%. It was also found in younger (≤20 years) apple orchards the occurrence of ACLSV decreased with the increase of tree age, but when trees were more than 20 years old, the occurrence of ACLSV increased. This is the first extensive survey in the last decade in China for monitoring ACLSV, which provides important information for ACLSV control in China.

Introduction

Latent viral infections are serious on apple (*Malus x domestica* Borkh.) and distributed worldwide. *Apple chlorotic leaf spot virus* (ACLSV) is one of the most important latent viruses, which causes significant yield reduction on commercial apple cultivars (Schmidt 1972; Wang et al. 2011). It is necessary to use virus-free seedlings or planting material to prevent virus spread and reduce the cost of production. In many countries, certification schemes were established to select healthy plants for propagation (Massart et al. 2008), but not yet developed in China. Apple is one of the most important and widely grown fruit crops in China. Therefore, the risk that virus has spread uncontrolled in apple orchards in infected planting material is very high. Previous studies on ACLSV occurrence in China were carried out in 1980s (Liu and Wang 1989), which was only on visual observations and biological indexing. During the last decade, many new cultivars have been introduced and new commercial orchards have been widely

established in China. There were no accurate data on the occurrence of ACLSV in apple orchards.

Infection by ACLSV on most cultivars of apple is usually symptomless. Therefore, an accurate and sensitive method is needed to detect the virus infection. The most common techniques for diagnosis of ACLSV are DAS-ELISA, woody indicator and RT-PCR, respectively. But DAS-ELISA often fails because of low sensitivity and the inhibitory effects of compounds in the woody plants. Use of woody indicators is time consuming, expensive, and the results sometimes are difficult to interpret (Nemchinov et al. 1995; Svoboda and Polák 2010). Therefore, RT-PCR techniques provide a possible alternative, which is more sensitive and can be used efficiently to process large numbers of samples.

ACLSV (the type species of the genus *Trichovirus*, family *Betaflexiviridae)* is characterized by having elongated, flexuous particles of 720 × 12 nm, encapsidating a single-stranded, positive sense genomic RNA (Liberti et al. 2005). Thirteen complete nucleotide sequences of ACLSV have been reported from apple (P205, A4, B6, AC-ind and MO-5) (German et al. 1990; Sato et al. 1993; Yaegashi et al. 2007), peach (TaTao5, Z1 and Z3) (Niu et al. 2012), cherry (Bal1) (German et al. 1997) and plum (P863, ACLSV and PBM1) (German et al. 1990; Jelkmann 1996). The genome of ACLSV, a 7474–7561 nt polyadenylated, single-stranded, plus-sense RNA, which contains three overlapping open reading frames (ORFs 1, 2 and 3). The 216-kDa replication-associated protein (Rep) is encoded by ORF1. The 50-kDa movement protein (MP) is encoded by ORF2, and the 21-kDa coat protein (CP) is encoded by ORF3 (Sato et al. 1993).

We have determined the nearly complete nucleotide sequences of ACLSV from apple in Baoding city of Hebei province, China. This is the first report of a China isolate of ACLSV genome sequence from apple. The occurrence and distribution of ACLSV in China were also surveyed by RT-PCR.

Materials and Methods

Viruses and plant materials

The MS isolate of ACLSV was collected from an infected apple tree in Baoding city. After identification by DAS-ELISA (Bioreba AG, Reinach, Switzerland) and RT-PCR, the isolate was maintained on *Chenopodium amaranticolor* plants in an insect-proof glasshouse. To evaluate the distribution of the ACLSV in China, 327 one-year-old branch samples (40 different cultivars of apple) were collected randomly from apple orchards in 13 provinces in China (Heilongjiang, Liaoning, Hebei, Beijing, Henan, Shanxi, Shaanxi, Shandong, Gansu, Ningxia, Xinjiang, Sichuan and Yunnan) during 2011. The samples were stored at −80°C until extraction of nucleic acid. The healthy samples pretested by DAS-ELISA and RT-PCR were used as the negative control.

Extraction of total nucleic acids and RT-PCR assay

Extraction of total nucleic acids and RT-PCR assay was according to the method described originally by Kumar et al. (2012). The primer sequence, primer position and expected amplified fragment size are given in Table 1. Total nucleic acids were extracted using bark chips containing vegetative buds 0–5 cm from the apex of 1-year-old branches. RT-PCR was carried out in a 1000^{TM} Thermal Cycler (BIO-RAD, Hercules, CA, USA). The PCR products (8 μl) were examined by electrophoresis in 1% (w/v) agarose gels in 1 × TAE buffer (40 mM tris-acetate, 1 mM EDTA) for 40 min at 120 V and stained with ethidium bromide. Reactions with and without a target template were included in every experiment as a positive and blank control, respectively.

Primer designing, molecular cloning and sequencing for the genome of ACLSV-MS

The genome sequences of ACLSV-MS were obtained using a series of primers (Table 2), which were based on the conserved region in the published genomes of ACLSV isolates. Successful amplification of the expected nucleotide segments was confirmed by electrophoresis in 1% (w/v) agarose gels. The PCR products were then purified by the DNA gel extraction kit (TaKaRa, Dalian, China) and cloned into the pMD18-T vector (TaKaRa) according to the manufacture's manual. The recombinant vectors were transformed into *Escherichia coli* strain DH5α. Plasmid DNA was

Table 1 Properties of the RT-PCR primers used for *Apple chlorotic leaf spot virus* (ACLSV) on apple in China

Primer name	Sequence (5′–3′)	Position[a]	Product size (bp)	Reference
ACLSV-F	CAGACCCCTTCATG GAAAGACAG	6860–6880	645 bp	Kumar et al. (2012)
ACLSV-R	TGACTCTTTATACT CTTTCATGGGTTC	7507–7536		

R, antisense primer; F, sense primer.

[a]The reference accession number (National Center for Biotechnology Information) for the determination of the primer positions is D14996.

Table 2 The primers used to determine the nucleotide sequences of ACLSV-MS isolate

Primer name	Sequence (5′–3′)	Position[a]
prAC1-F	CAGATTGACGTAACGCCTCAATC	38–60
prAC1-R	TCCATATGCATCTTAAGCAT	3572–3795
prAC2-F	GTCCAGGAATTGAACTTCTCG	3284–3304
prAC2-R	GGAATATCCCCTTCTGCGAT	5754–5773
prAC3-F	GGTGAGAGGCTCTATTCACATCTTG	5579–5603
prAC3-R	TCACACACCTGGCGGAAAGTCATG	7077–7100
prAC4-F	ATCGCAGAAGGGGATATTCC	5754–5773
prAC4-R	GGCTATTTATTATAAGTCTAAACAC	7503–7527
prAC5-F	ATGGCGGCAGTTCTGAATTTACAGC	6784–6808
prAC5-R	CTAAATGCAAAGATCAGTTGTAAC	7342–7365

[a]The reference accession number (National Center for Biotechnology Information) for the determination of the primer positions is D14996.

obtained from colonies selected from overnight cultures by alkaline lysis. Shanghai Sangon Biotech Company (Shaihai, China) sequenced the cloned fragments. At least three clones were sequenced for each segment. The sequences were compared with the corresponding virus sequence retrieved from the GenBank database.

Sequence and phylogenetic analysis

Sequence segments were assembled by Vector NTI, version 10.0 software (Informax, Frederick, MD, USA). Other genome sequences were retrieved from NCBI, and multiple alignments were made using the Clustal w algorithm in dnaman (Lynnon Corp., Pointe-Claire, QC, Canada). Twelve ACLSV sequences (Table 3) were used for comparison or phylogenetic tree construction using a neighbour-joining of Bootstrap Test in mega5.0 (version 5.0; Koichiro, Tokyo, Japan). *Apricot pseudo chlorotic leaf spot virus* (APCLSV) and *Peach mosaic virus* (PeMV) were used as outgroup control.

Results

Molecular characterization of ACLSV-MS

The nearly complete genome of ACLSV-MS consisting of 7376 nt has been submitted to GenBank (Accession No. KC847061). Approximately 166 nt were not obtained as with other ACLSV isolates. The ACLSV-MS genome contains three ORFs and two untranslated regions (UTRs) organized similarly to the other 12 ACLSV isolates. ORF1 encodes the 216.4 kDa Rep (nt 4–5652). ORF2 encodes the 50.8 kDa MP (nt 5573–6946) and ORF3 encodes the 21.5 kDa CP (nt 6633–7211). It also has three nucleotides of uncompleted 5′ UTRs and 165 nucleotides of 3′ UTRs, respectively.

An alignment was made between ACLSV-MS and the 12 ACLSV isolates as well as APCLSV and PeMV

Table 3 Comparison of the nearly complete genome sequence and different ORFs of ACLSV-MS isolate in China to the 12 *Apple chlorotic leaf spot virus* (ACLSV) isolates reported previously: ACLSV-Balaton1 (X99752), ACLSV-MO-5 (AB326225), ACLSV-TaTao5 (EU223295), ACLSV-B6 (AB326224), ACLSV-P863 (M58152), ACLSV-PBM1 (AJ243438), ACLSV-Z1 (JN634760), ACLSV-Z3 (JN634761), ACLSV-A4 (AB326223), ACLSV-AC-ind (HE980332), ACLSV-P-205 (D14996), ACLSV (NC_001409), APCLSV (NC 006946) and PeMV (NC 011552)

			Nearly whole genome		ReP gene		Movement protein gene		Coat protein gene	
Virus isolate	Country	Host	nt	nt%	nt	nt%	nt	nt%	nt	nt%
ACLSV-MS	China	Apple	7376	–	5649	–	1374	–	579	–
ACLSV-Balaton1	France	Cherry	7379	76.8	5661	75.9	1374	80.3	578	84.3
ACLSV-MO-5	Japan	Apple	7387	76.1	5631	74.8	1374	81.4	579	84.5
ACLSV-TaTao5	USA	Peach	7290	69.5	5640	70.2	1344	68.2	579	71.2
ACLSV-B6	Japan	Apple	7380	82.3	5646	80.9	1374	86.3	579	91.5
ACLSV-P863	France	Plum	7382	80.2	5652	79.1	1374	83.4	579	86.4
ACLSV-PBM1	Germany	Plum	7372	80.3	5649	79.3	1374	84.1	579	86.2
ACLSV-Z1	China	Peach	7379	79.6	5649	78.4	1374	83.8	579	88.4
ACLSV-Z3	China	Peach	7379	79.7	5649	78.5	1374	83.3	579	89.5
ACLSV-A4	Japan	Apple	7375	80.7	5655	79.8	1374	84.1	579	86.5
ACLSV-AC-ind	India	Apple	7378	81.5	5646	80.5	1374	84.3	579	90
ACLSV-P-205	Japan	Apple	7379	81.4	5655	81.1	1371	83	579	85.3
ACLSV	France	Plum	7382	80.2	5652	79.1	1374	83.4	579	86.4
APCLSV	Italy	Plum	7390	65.9	5676	66.4	1365	66.6	579	69.1
PeMV	USA	Peach	7318	63.6	5676	65.3	1365	60.1	579	59.7

as outgroup. Results showed (Table 3) that the nearly full genome sequence of ACLSV-MS shared 69.5–82.3% identities with other ACLSV isolates described previously, 70.2–81.1% for Rep gene, 68.2–86.3% for the MP gene and 71.2–91.5% for the CP gene. ACLSV-MS shared the greatest sequence identity with ACLSV-B6 from apple for nearly the whole genome, MP and CP, except for Rep (P-205 from apple) and the least sequence identity with ACLSV-TaTao5 from peach not only for the nearly whole genome but also for all the three genes. MS isolate shared only 65.9 and 63.6% identity with two other members of the genus *Trichovirus:* APCLSV and PeMV. The result of alignments showed that there was a high variability, at the molecular level, between the different ACLSV isolates, and the nt sequences encoding CP were the most conserved, and nt sequences encoding MP were the least conserved.

A phylogenetic tree based on nucleotide sequence alignments (Fig. 1) confirmed that there was high molecular variability in the genome of ACLSV. The isolates from apple formed one cluster, including MS, P205, B6, A4, AC-ind, except for the MO-5 isolate. ACLSV-P205 and B6 were closer to MS than other isolates. PeMV and APCLSV belong to a separate clade in the same subcluster.

Incidence and distribution of ACLSV on apple in China

RT-PCR was used to detect ACLSV in 327 field apple samples collected from 13 provinces (56 sites) of China during the growing season of 2011. Two hundred and twenty-eight samples (69.7%) reacted positively. To determine the reliability of the method, 50 field samples randomly selected from 327 samples were tested by DAS-ELISA. The numbers of samples tested positive were consistent with the results obtained by RT-PCR assay (data not shown).

The incidence of ACLSV in field samples of apple is shown in Table 4. The virus was present in all regions surveyed (Heilongjiang, Liaoning, Hebei, Beijing, Henan, Shanxi, Shaanxi, Shandong, Gansu, Ningxia, Xinjiang, Sichuan and Yunnan). The results suggested that the virus is widely spread in China. The occurrence was most serious in Heilongjiang province (10 samples) with an incidence of 100% followed by Henan (30 samples) province with an incidence of 86.7%. The occurrence was relatively lower in Liaoning (20 samples) and Shanxi (10 samples), with an incidence of 50%.

The occurrence of ACLSV on different apple cultivars in China

Fuji, Ralls, Gala, Golden Delicious and Starking are common and economically important apple cultivars in China. The occurrence of ACLSV on the five cultivars is shown in Fig. 2. Cv. Gala (10 samples) had the highest incidence of 33.3%, followed by cv. Golden Delicious (13 samples) with an incidence of 23.1%. The incidences were 22.8, 19.2 and 18.2% on cvs Fuji (167 samples), Ralls (26 samples) and Starking (22 samples), respectively.

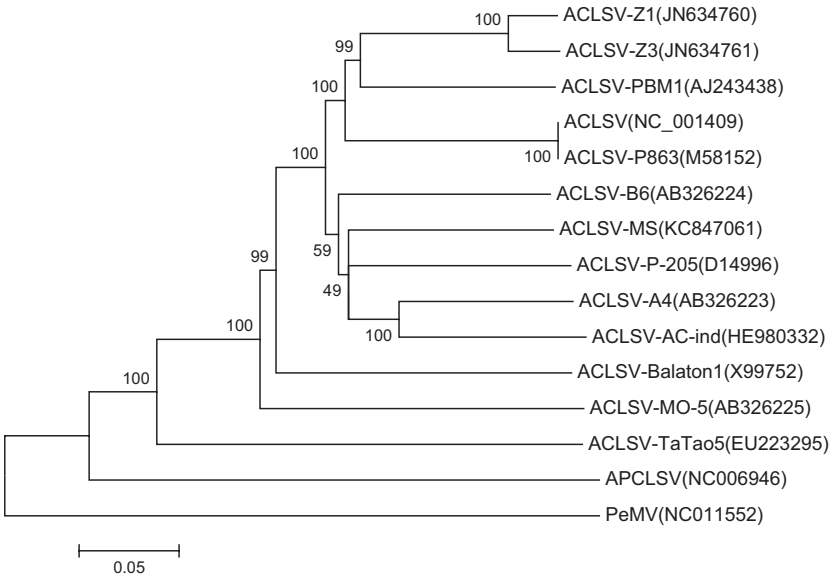

Fig. 1 Phylogenetic tree constructed by alignment of the nearly complete nucleotide sequences of *Apple chlorotic leaf spot virus* (ACLSV) of 15 isolates. Statistical reliability of the nodes was obtained by bootstrap analysis (1000 replications). Phylogenetic analysis was conducted by neighbour-jointing method of MEGA5.0. Numbers on branches are percentages of bootstrap analysis supporting the grouping of each branch. The isolates were including ACLSV-MS (KC847061), ACLSV-AC-ind (HE980332), ACLSV (NC_001409), ACLSV-Z1 (JN634760), ACLSV-Z3 (JN634761), ACLSV-P863 (M58152), ACLSV-TaTao5 (EU223295), ACLSV-B6 (AB326224), ACLSV-MO-5 (AB326225), ACLSV-Balaton1 (X99752), ACLSV-P-205 (D14996), ACLSV-PBM1 (AJ243438), ACLSV-A4 (AB326223), APCLSV (NC 006946) and PeMV (NC 011552).

Table 4 Detection of 327 samples of bark tissue from 40 different apple cultivars from 13 provinces for *Apple chlorotic leaf spot virus* in China

Collection areas	No. of sites	Tree age (years)/no. of samples/ cultivars	Positive (%)
Heilongjiang	1	2, 3, 5, 8, 10/10/Jinhong, Longfeng, Longguan	100
Liaoning	3	6, 10, 11, 12, 14, 18, 20, 23/20/ Jonathan, Wangjianghong, Sansa, Golden Delicious, Liangxiang, Alps Otome, Gaolonghanfu, J ieke11, Fuji, Orin	50
Hebei	15	1–10, 12–14, 18, 20, 23–25/109/Fuji, Starking, Ralls, Shengli, Golden Delicious, Sansa, Xinshiji, Danxia, Shinsckai, Jonagold, Red Delicious	67.9
Beijing	3	4, 18, 23, 27/10/Fuji	60
Henan	8	2, 4, 5, 6, 8, 15, 18, 21, 23, 26, 28/30/ Bene Shogun, Fuji, k12, Balenghaitang, Gala, Yuhuazaofu, Starking	86.7
Shanxi	2	17, 20/10/Fuji	50
Shaanxi	7	5, 8, 16, 17, 18, 24, 25/44/Qinguan, Fuji, Qianqiu, Gala, Red Fuji, Xinnonghong, Lifu, Micui	65.9
Shandong	5	2, 7, 8, 10, 12, 14, 15/20/Bene Shogun, Nanfangcui, Yoko, Honglu, Weixi, Hongtailang, Ralls	65
Gansu	2	2, 3, 4, 5, 6, 2, 10, 12, 15/20/Starking, Fuji	60
Ningxia	1	3, 4, 6, 18, 23/10/Matsumoto Nishiki, Fuji, Ningguan, Gala, Golden Delicious	70
Xinjiang	1	10/20/Golden Delicious, Fuji, Gala	70
Sichuan	5	1, 4, 5, 6, 10,12,13/10/Fuji	70
Yunnan	3	3, 12, 15/14/Fuji, Golden Delicious	57.1
All samples	56	1–28/327	69.7

The occurrence of ACLSV on apple of different tree age

All the 327 samples were divided into five different types according to tree age, including 1–5, 6–10, 11–15, 16–20 years and more than 20 years. The occurrence of ACLSV on apple trees of different ages is shown in Fig. 3. The occurrence was most serious on the stage of 1–5 years (65 samples) with an incidence of 31% followed by more than 20 years (43 samples) with an incidence of 21%. The incidence rates were 27, 21 and 13% on the stage of 6–10 years (96 samples), 11–15 years (53 samples) and 16–20 years (70 samples).

Discussion

Apple (*Malus x domestica* Borkh.) is one of the most important and widely grown fruit crops in China. The

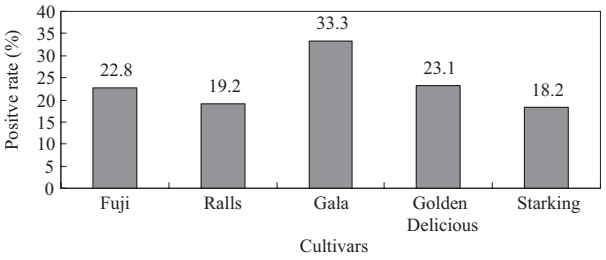

Fig. 2 Occurrence of *Apple chlorotic leaf spot virus* on five common cultivars of apple in China.

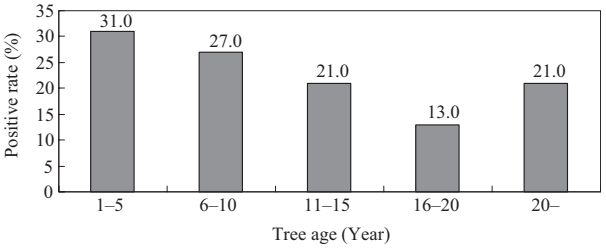

Fig. 3 Occurrence of *Apple chlorotic leaf spot virus* on different tree age of apple in China.

planted areas are 1961.8 thousand ha in 2007 (data from website of MOA). ACLSV is an important virus on apple in the country (Liu and Wang 1989). We report, for the first time, a China isolate of ACLSV genome sequence from apple and made comparisons of the nucleotide sequence with other ACLSV isolates. Based on the phylogenetic analysis of complete genome, we proposed that two types of ACLSV isolates may exist in apple, the MS type and the MO-5 type. ACLSV-MS had relative high nucleotide sequence identity with ACLSV-P205 and ACLSV-B6, both of which were found in Japan. The development of apple import and export trade between Japan and China might be the reason, which could introduce the virus into China. ACLSV-MS and the two isolates are included in the same group, indicating that the two isolates may be evolutionarily closer than other isolates.

An extensive survey was conducted in our study using RT-PCR. 327 samples were collected from 13 provinces and 40 different commercial cultivars of China, which represented a wide geographical distribution and host cultivars. The result showed that ACLSV was spread in most apple orchards and most commonly grown cultivars in China, which indicate that infected planting material was used in propagation, because only grafting is the main transmission mode for ACLSV, and the natural spread is usually

slow (Fritzsche and Kegler 1968; Lister 1970). So, establishment of a certification programme or the implementation of a clean stock programme to prevent the occurrence of the virus needs immediate attention.

We also found, in younger (≤20 years) apple orchards, the occurrence of ACLSV was reduced with the increase in tree age, once the tree age was more than 20 years, the occurrence of ACLSV increased. Probably because a virus-free certification scheme has not been developed in China, the percentage of clonal rootstocks and scions from infected mother trees is increasing year by year. The risk of young fruit trees planted in recent years being infected with ACLSV is relative higher. While the fruit trees more than 20 years old have such poor resistance to the virus that the risk being infected increases.

The occurrence of ACLSV in five common cultivars was also tested regardless of the rootstock. The selection of the cultivars was based on their common use by growers in the country. The carrying percentage of ACLSV was highest in cv. Gala, and lowest in cv. Starking, which maybe indicate that cv. Gala is more susceptible and cv. Starking is more resistant to the virus. The results need to be confirmed by further study because of the limited number of samples. In other studies, similar results were obtained. For example, Yardimci and Eryigit (2005) reported that a total of 276 samples were collected from apple cvs Golden Delicious, Starking Delicious, Granny Smith and Imperatore and tested for *Apple mosaic virus* (ApMV); 82 samples of cvs Granny Smith and Imperatore were infected with ApMV, but none of cvs Golden Delicious and Starking Delicious. Africander and Siebert (1999) reported that cvs Jonathan and Golden Delicious were more susceptible to ApMV, and cvs Bon Chretien, Packham's Triumph and Forelle to *Apple stem grooving virus* (ASGV). To our knowledge, there is no report about the susceptibility of apple cultivars to ACLSV. Our results could provide valuable information for others.

In conclusion, we have reported the almost complete nucleotide sequence and the characterization of the genome of a Chinese isolate of ACLSV from apple (ACLSV-MS, GenBank accession number KC847061). The virus occurred in most apple orchards and most commonly grown cultivars in China, with an average incidence of 69.7% and the incidence of ACLSV increased in the younger orchards, which alarmed us there is a great need for the establishment of sanitary and certification system for apple tree propagation in China.

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