A TaqMan-based Quantitative RT-PCR Method for Detection of *Apple Chlorotic Leaf Spot Virus* in Hawthorns

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Abstract*—ACLSV is one of the major fruit viruses and can cause severe diseases in species of family Rosaceae. Previous RT-PCR methods are available to detect ACLSV in hawthorn samples, but not to evaluate the infected level of ACLSV. In this study, a TaqMan-based quantitative RT-PCR detection method targeting CP gene of ACLSV was first established and the sensitivity and reproducibility were investigated. The results indicated that this standard curve between log of plasmid DNA concentration versus the cycle threshold (Ct) value generated a linear fit with a linear correlation (R^2) of 0.99 and the PCR efficiency was more than 90%. The quantitative RT-PCR method was high sensitive and able to detect 6.9 × 10^2 copies·μL^{-1} of ACLSV RNA. Compared with the conventional RT-PCR method, it was 100-fold sensitive in detection of ACLSV. In addition, different organs of hawthorn samples were examined using the quantitative RT-PCR repeatedly and the result revealed that the quantitative RT-PCR is not only an effective detection method, and can obtain an absolute quantitation for ACLSV.*

***Keywords*—TaqMan, quantitative RT-PCR, ACLSV, detection, sensitive.**

I. INTRODUCTION

ACLSV is the type species of genus *Trichovirus* in family *Betaflexiviri*dae [1]. ACLSV causes a severe decline in trees and the quality of fruits, is transmitted by the grafting, pruning, or propagation of materials and nematodes, and has an extensive host range of most fruit tree species of family *Rosaceae*, including apple, pear, peach, plum, almond, apricot, cherry and hawthorn [2]. The virus genome is composed of 7474–7561 nucleotides, excluding poly-adenylated tails. It contains three overlapping open reading frames (ORFs) that encode a 216-kDa replication-associated protein (Rep), a 50-kDa movement protein (MP), and a 22-kDa coat protein (CP) [3-4].

The coat protein is the only constitutive protein, although this region is the most conserved part of the genome, sequence diversity has been reported among many isolates [5-6]. Different isolates have great genetic diversity and molecular variation of ACLSV in China and the world. A recent study showed that ACLSV variants in the world clustered separately were unrelated to host species or geographic origin, while natural recombinant events among isolates/genotypes play a role in ACLSV evolution [7]. Recently, mixed infections by ACLSV and *Apple stem grooving virus* (ASGV) and/or *Apple stem pitting virus* (ASPV) in the same tree have been reported [8]. These viruses are transmitted with infected propagating materials. Planting virus-free propagating materials is a main method to prevent and cure virus disease, while, it first demands an accurate, rapid and reliable virus detection method.

Conventional virus detection methods of ACLSV are based on virus isolation and serological tests, both of which are time consuming and laborious [9]. Nucleic acid based RT-PCR has been recommended for detection ACLSV because of its sensitivity, specificity and less time consuming, however the sensitive of this method remains a question when virus titer is low in plant samples and it is prone to sample contamination occurring in gel electrophoresis, which increases incidence of false-positive results [10-12]. More recently, a TaqMan-based quantitative RT-PCR method is established that allows accurate and reproducible quantitation for the detection of fruit trees viruses. The quantitative RT-PCR improves diagnosis sensitivity (10-1000 fold more sensitive than PCR/RT-PCR), practicality, reduces risk of carry-over contaminations and can quantitate estimation of viral load [13].

In this study, we proved the quantitative RT-PCR method developed here is a highly efficient and practical method for the detection of ACLSV in hawthorn. Since viral genomes have a relatively high mutation rate and even few nucleotide mismatches between the target sequences and the primers or the probe may lead to false negative results, therefore, the TaqMan primers and probe used in in our research were designed to fit the most conservative CP genome sequence. Standard curve from a plasmid that contained a CP gene sequence of ACLSV were used to obtain an absolute quantitation of ACLSV. The sensitivity and reproducibility of this method were investigated. In addition, this is first report on the quantitative RT-PCR method that applied to detect ACLSV and to evaluate the virus present in hawthorn samples.

II. MATERIAL AND METHOD

2.1 Plant material

Hawthorn *(Crataegus pinnatifida)* accession used in this study, which was infected with ACLSV isolate SY01[6], was kept in the National Hawthorn Germplasm Repository in Shenyang.

2.2 RNA extraction and cDNA synthesis

Total RNA of hawthorn was extracted from leaves by magnetic bead RNA extraction machine produced by Thermo Fisher Scientific. The sample was 100 mg, and the amount of buffer was 30 μL. Agarose gel electrophoresis was applied to analyze RNA quality and integrity, and NanoDrop 2000 ultraviolet spectrophotometer was used to test the concentration and purity of RNA. Reverse transcription reactions were performed at 37 °C for 30 min, then 85 °C for 5 s with PrimeScript® RT reagent kit (TaKaRa, Japan) according to the manufacturer's instructions.

2.3 TaqMan primers and probe design

TaqMan primer pairs and probe for ACLSV were designed based on the partial conserved CP gene nucleotide sequence of ACLSV hawthorn isolates published in the GenBank database (accession no. KM207212). The sequence of the forward primer F2 is 5′- AGACACTGGAGTCCATCTTCGC -3′ and of the reserve primer R2 is 5′- CAGGCATGGTTGTGAAGAG GT-3′. The fluorescent dye-labeled TaqMan probe sequence P: 5′- FAM-TAGCGATCCAGGGAACTTCGGAGCAG-TAMRA -3′. A 294 bp product was amplified when the primers were used. The primers and probe used for TaqMan quantitative RT-PCR were designed by the Primer 5.0 software and synthesized and labeled by TaKaRa.

2.4 Preparation of the standard plasmid DNA

The CP gene fragment of ACLSV was amplified by PCR using the following pair of primers: forward primer F1, 5′-CCCGGG-AATGGCAGCAGTTTTAAATCTT-3′, reserve primer R1, 5′-GCGGCCGC-TTAAATGCAAAGATCAGTCGT-3′, which added the enzyme digestion sites *Sma*I and *Not*I, respectively. PCR was performed in 20 μL total volumes with reaction mixtures that contained 1 μL cDNA, 1.6 μL of each dNTP (2.5 mM), 2.0 μL 10× PCR buffer, 1.0 μL $MgCl_2$ (25 mM), 0.5 μL of each primer (10 μM), 1 U Taq DNA polymerase (Promega, USA) and ddH_2O to yield a 20 μL final volume. After the RT-PCR reaction, agarose gel electrophoresis was used to test the reaction product. PCR products were gel-extracted and ligated into a pMD18-T vector (TaKaRa, Japan). Positive clones for each product were sequenced at Beijing Genomics Institute, China. The recombination plasmid, named pMD18-ACLSV-CP, was purified using a commercial test kit (OMEGA, China). The concentration of pMD18-ACLSV-CP plasmid was tested by the NanoDrop 2000 ultraviolet spectrophotometer, and the number of copies of pMD18-ACLSV-CP was calculated using the following formula: copy number (copies·μL^{-1}) = (6.02×10^{23} copies) × (plasmid concentration ng·μL^{-1}) / (numbers of base) × (660 daltons·$base^{-1}$).The plasmid was maintained at -20℃ for use as standard DNA in subsequent experiments.

2.5 Conditions optimization for quantitative RT-PCR method

The quantitative RT-PCR method was established using the Applied Biosystems® 7500 Real Time PCR System and TIANGEN RealMasterMix (Probe). This detection method was optimized by varying concentrations of the primers and probe to obtain the lowest Ct value with higher fluorescence intensity. Keeping other factors unchanged, eight concentrations of primers were used, the initial concentration was 100 nM, rising 100 nM until 800 nM, with three replicates in each group. The most optimal concentration was selected based on the results of the method, and using that concentration, eight concentrations of probe were tested, the initial concentration was 100 nM, rising 50 nM until 450 nM, with three replicates in each group.

2.6 Establishing a standard curve for quantitative RT-PCR

The standard curve for quantitative RT-PCR was performed with seven serial dilutions of the standard plasmid DNA (concentrations ranged from 10^8 copies·μL^{-1} to 10^2 copies·μL^{-1}). The quantitative RT-PCR reaction contained 2.5 × realMasterMix 8.0 μL, 400 nM of each primer, 300 nM TaqMan probe and 1 μL template plasmid DNA in a total volume of 20 μL by using the following cycling condition: 95 °C for 10 min, 40 cycles at 95 °C for 30 s, and 60 °C 1 min. The method was repeated at least 3 times with each template and the negative control.

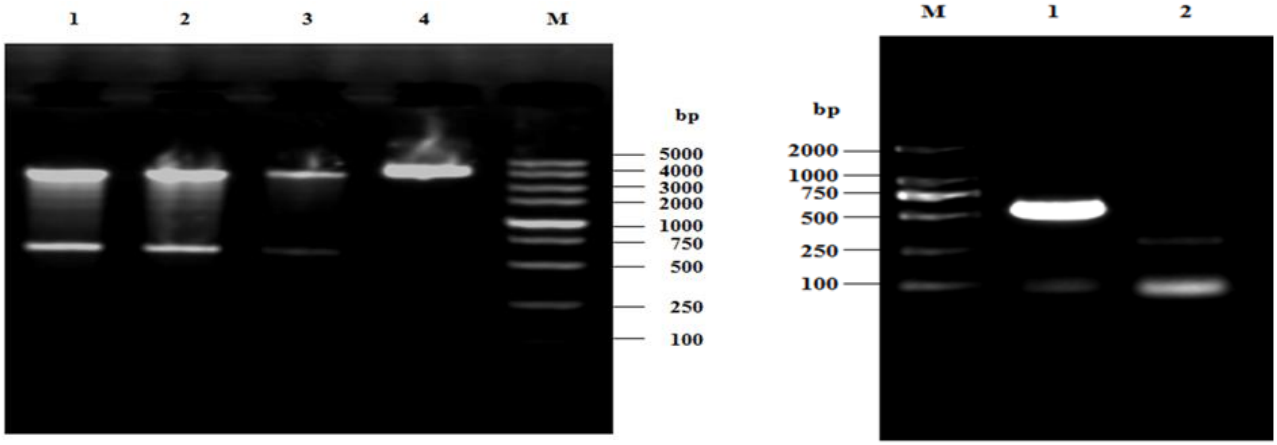
2.7 Evaluation of the method

The sensitivity of the real-time quantitative RT-PCR and conventional RT-PCR method for the detection of ACLSV was tested using the pMD18-ACLSV-CP plasmid DNA diluted serially 10-fold from 10^{10} copies·μL^{-1} to 10^{0} copies·μL^{-1} with sterile water. The quantitative and conventional RT-PCR was performed as described in the front sections. The method was repeated at least 3 times with each template and the negative control. The reproducibility of the ACLSV TaqMan quantitative RT-PCR method was assessed by calculating the CV values of the Ct values obtained after amplification of 10-fold serial dilutions of the pMD18-ACLSV-CP plasmid ranging from 10^{7} to 10^{5} copies per reaction in triplicate during the same experiment. Different organs of hawthorn samples, including young leaves, mature leaves, flower, shoot, young fruit (15 DAB), from infected ACLSV were tested to determine the practicability and feasibility of the TaqMan RT-PCR method. At the same time, these samples were subjected to conventional RT-PCR repeatedly.

III. RESULTS

3.1 Identified of the recombinant plasmid

The recombinant plasmid was identified by enzyme digestion. After the digestion reaction, agarose gel electrophoresis was used to test the digestion product. As showed in (Fig. 1A), a fragment of the expected size (597 bp) was observed, with the sequencing data, which indicated the recombinant plasmid contained the target CP gene sequences. In (Fig. 1B), with quantitative primers F2/R2, the virus' specific fragments of the expected size (294 bp) were amplified.



(A) **(B)**

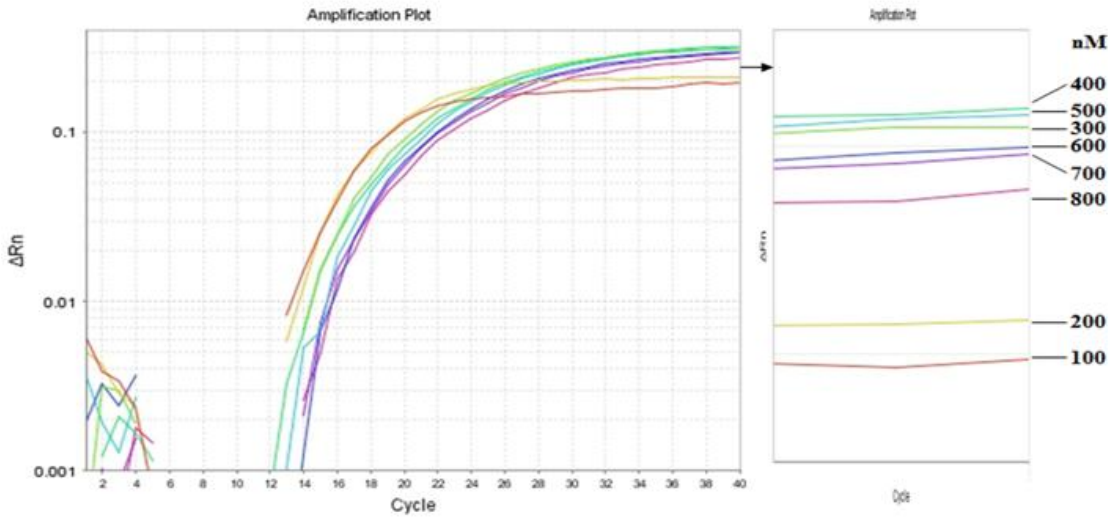
FIG. 1. IDENTIFIED OF THE RECOMBINANT PLASMID. (A)LEFT. AGAROSE GEL ELECTROPHORESIS OF THE RECOMBINANT PLASMID DIGESTED WITH RESTRICTION ENZYME. M REPRESENTS THE DL5000 MARKER, 1-3 REPRESENT THE DOUBLE ENZYMATIC DIGESTION PRODUCT OF pMD18-ACLSV-CP, 4 REPRESENT THE RECOMBINANT PLASMID OF pMD18-ACLSV-CP. (B)RIGHT. PCR-IDETIFIED RESULT OF THE RECOMBINANT PLASMID. M REPRESENTS THE DL5000 MARKER, 1-2 REPRESENT PCR RESULT OF POSITIVE RECOMBINANT PLASMID USING PRIMER F1/R1 AND F2/R2, RESPECTIVELY.

3.2 Optimization of quantitative RT-PCR method parameters

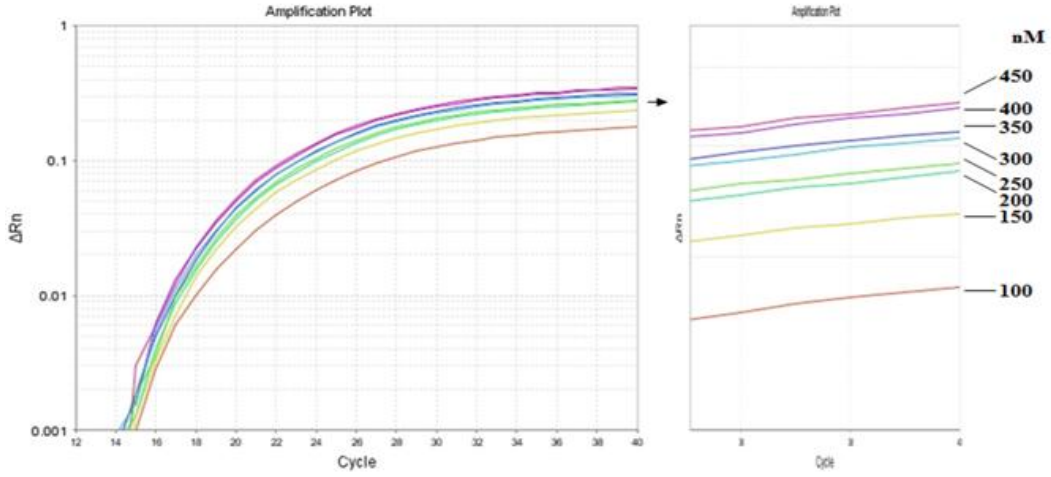
The strongest fluorescence was observed when the concentrations of primer and probe were 400 nM and 450 nM, respectively (Fig. 2). However, the fluorescence is enough for detection when the concentration of probe was 300 nM (Fig. 2B). So, the optimized quantitative RT-PCR reaction volume was 20μL contained 2.5 × realMasterMix 8.0 μL, 400 nM of each primer, 300 nM TaqMan probe and 1 μL template plasmid DNA. The optimized cycling conditions were 95 °C for 10 min, 40 cycles at 95 °C for 30 s, and 60 °C 1 min.

3.3 Establishing a standard curve for quantitative RT-PCR

The standard curve was generated using different template concentrations ranging from 6.9×10^{8} to 6.9×10^{2} copies·μL^{-1} obtained by 10-fold serial dilutions. This curve between log of DNA concentration versus Ct value generated a linear fit with a slope of -3.18 and linear regression coefficient (R^2) of 0.99 and the PCR efficiency was more than 90% (Fig. 3)



(A)



(B)

FIG. 2. (A) OPTIMIZATION OF THE PRIMER CONCENTRATION. THE PRIMER CONCENTRATION IS RANGING FROM 100-800 NM. (B) OPTIMIZATION OF THE PROBE CONCENTRATION. THE PROBE CONCENTRATION IS RANGING FROM 100-450 NM.

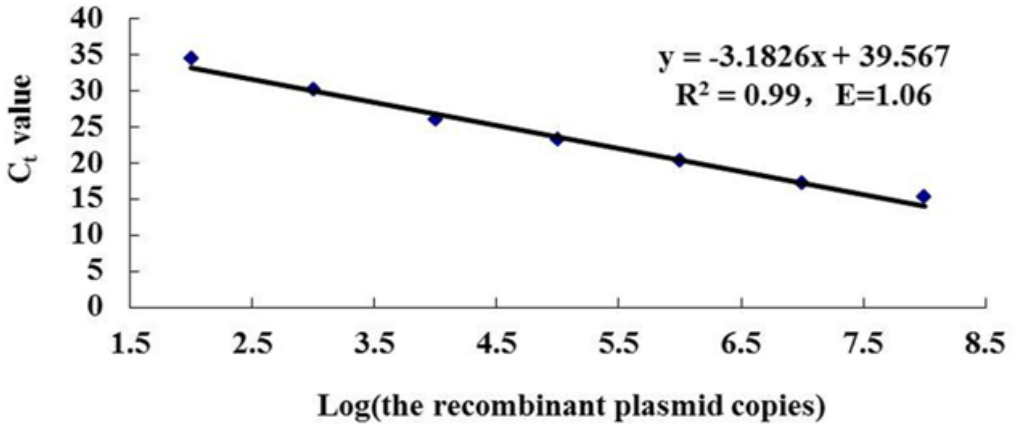


FIG. 3. THE STANDARD CURVE OF QUANTITATIVE RT-PCR TO DETECT THE ACLSV RECOMBINANT PLASMID. NOTE, STANDARD CURVE WAS OBTAINED WITH SERIAL DILUTION OF THE RECOMBINANT PLASMID FROM 10^2 TO 10^8 COPIES.

3.4 The quantitative RT-PCR sensitivity and reproducibility

The quantitative RT-PCR for detection of ACLSV showed high sensitivity, the detection limit was 6.9×10^2 copies·μL^{-1} (Fig. 4), while the conventional RT-PCR method showed positive results only when more than 6.9×10^4 copies·μL^{-1} template were used (Fig. 5). Therefore, the sensitivity of the quantitative RT-PCR is 100 times higher than that of conventional RT-PCR. According to the coefficients of variation (CV) between intra- and inter- Ct value, we evaluated the repeatability of the quantitative RT-PCR method. When the 3 different concentrations of plasmids (ranging from 10^7 to 10^5 copies per reaction) were examined using the real-time RT-PCR repeatedly, the coefficients of variation (CV) values of both intra- and inter- method were less than 0.91 % (Table S1), indicating that quantitative method for the detection of ACLSV is stable and reliable.

3.5 Detection of ACLSV in different organs of hawthorn

In different organs and tissues of infected ACLSV plants, the content of virions is not same. In this study, total RNA was extracted from the different organs of hawthorn, including young leaves, mature leaves, flower, shoot, young fruit (15 DAB), respectively, for detecting ACLSV by semi-quantitative and real time quantitative RT-PCR methods. The detection results were compared with each other using the same quantitative primers sequences. As showed in (Fig. 6) and (Fig. 7), both the semi-quantitative and real time quantitative RT-PCR can be used to detect ACLSV in different organs of hawthorn. The sensitive of quantitative RT-PCR is higher than that of the semi-quantitative RT-PCR and the absolute quantity of virus copy number in different organs can be calculated.

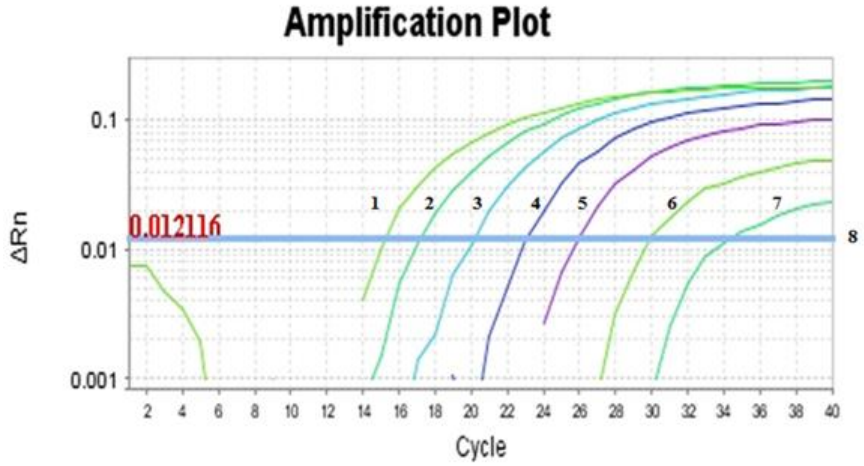


FIG. 4. THE SENSIBILITY OF REAL-TIME QUANTITATIVE RT-PCR FOR DETECTING THE RECOMBINANT PLASMID. 1-7 REPRESENT THE RECOMBINANT PLASMID OF 10^8~10^2 copies·μL^{-1}, 8 REPRESENT THE RECOMBINANT PLASMID OF 10^1~10^0 copies·μL^{-1} AND NEGATIVE CONTROL.

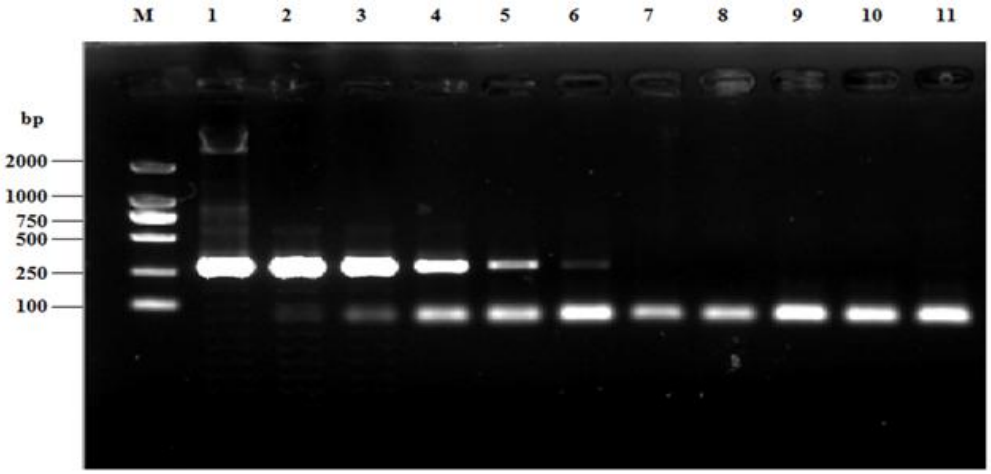


FIG. 5. THE SENSIBILITY OF CONVENTIONAL RT-PCR FOR DETECTING THE RECOMBINANT PLASMID. M REPRESENTS THE DL2000 MARKER, 1-10 REPRESENT THE RECOMBINANT PLASMID OF 10^9~10^0 copies·μL^{-1}, 11 REPRESENT THE NEGATIVE CONTROL.

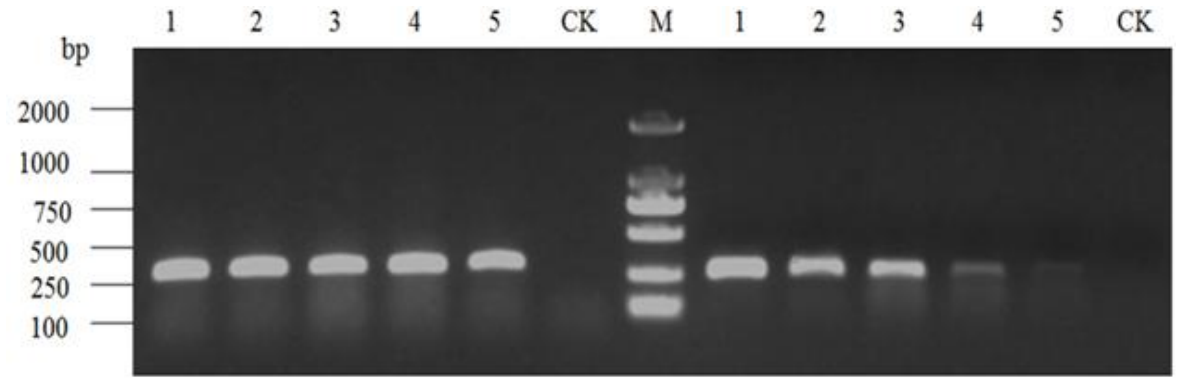


FIG. 6. DETECTION OF ACLSV IN DIFFERENT ORGANS OF HAWTHORN BY CONVENTIONAL RT-PCR. M REPRESENTS THE DL2000 MARKER, 1 REPRESENT YOUNG LEAVES, 2 REPRESENT MATURE LEAVES, 3 REPRESENT FLOWER, 4 REPRESENT SHOOT, 5 REPRESENT YOUNG FRUIT, CK REPRESENT NEGATIVE CONTROL WITH WATER AS TEMPLATE. LEFT REPRESENT ACTIN GENE, RIGHT REPRESENT ACLSV.

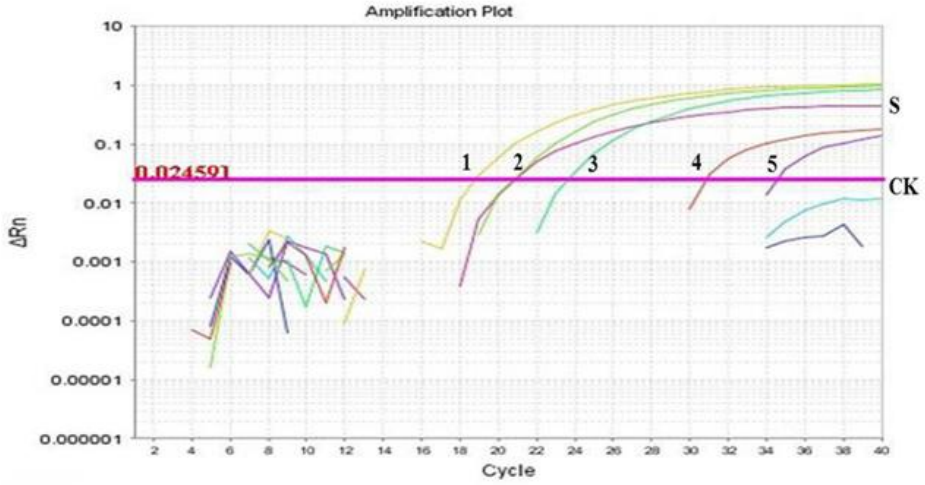


FIG. 7. DETECTION OF ACLSV IN DIFFERENT ORGANS OF HAWTHORN BY TAQMAN QUANTITATIVE RT-PCR. S REPRESENTS THE POSITIVE RECOMBINANT PLASMID, 1 REPRESENT YOUNG LEAVES, 2 REPRESENT MATURE LEAVES, 3 REPRESENT FLOWER, 4 REPRESENT SHOOT, 5 REPRESENT YOUNG FRUIT, CK REPRESENT NEGATIVE CONTROL WITH WATER AS TEMPLATE.

IV. DISCUSSION

ACLSV as one of economically important latent viruses can cause acute disasters in fruit quality and reduce the yields of fruit crop [14]. Also, it can produce different symptoms in sensitive fruit tree cultivars. Detection of viruses may contribute to control the globe spread of viruses through infected plants, propagation materials and postharvest products [15]. Moreover, the quantitative estimation of virus populations prevailing is essential, not only to predict disease outbreaks, but also for plant quarantine measures [16-17]. In addition, virus detection is best carried out as early as possible. So, an effective and available method for the rapid detection of ACLSV is essential.

Besides producing rapid results, quantitative RT-PCR has the advantage of simpleness and highly sensitivity for screening and virus quantification of large-scale samples compared to conventional RT-PCR. Our newly established quantitative RT-PCR method had an ACLSV detection limit of 100 copies, which is 100 times more sensitive than conventional RT-PCR. Ferriol et al. used RT-qPCR for detection and quantitation *Broad bean wilt virus* 1(BBWV-1) and BBWV-2, showing it also had a detection limit of 100 copies [18]. Diego et al. found that, the TaqMan real time RT-PCR for the rapid detection of *Raspberry leaf mottle virus* (RLMV) was 100 times more sensitive than conventional RT-PCR [19]. In experiments to detect four potato viruses by TaqMan real-time RT-PCR, Agindotan et al. found that the linear dynamic range of detection was 10^2 to 10^6 or 10^7 [20], which were consistent with the results obtained in this study.

In this study, a highly sensitive and practical method for the detection of ACLSV was developed. Since CP gene of ACLSV is among the most conserved regions and has been chosen as a preferred target region for the detection of ACLSV, the primers and probe were designed to amplify target sequences at the CP gene region of the ACLSV genome for the quantitative RT-PCR method. The method was high sensitive and able to detect 6.9×10^2 copies per reaction of ACLSV RNA. Compared with the conventional RT-PCR method, it was 100-fold sensitive in detection of ACLSV. In addition, the quantitative RT-PCR method, including RNA extraction, took approximately 3.5 h [21], which is faster than the conventional RT-PCR and other virus isolation method. In conclusion, this study is the first report on a TaqMan-based quantitative RT-PCR method that is able to detect ACLSV from Chinese hawthorn. This technique should be a quantitative, rapid, convenient, easy to finished and has the advantage for detecting large numbers of samples.

TABLE. S1
THE REPRODUCIBILITY OF THE POSITIVE RECOMBINANT PLASMID.

The recombinant plasmid copies (copies·μL^{-1})	Mean ± SD		CV (%)
	Intra-assay reproducibility	Inter-assay reproducibility	
6.9×10^7	16.08±0.06	16.78±0.06	0.37
6.9×10^6	19.03±0.16	19.86±0.18	0.84
6.9×10^5	22.00±0.04	22.86±0.16	0.18

V. CONCLUSION

A TaqMan-based quantitative RT-PCR method was established as an effective method for the rapid detection of ACLSV with sensitivity and practicability. This is the first report to illustrate the application of an absolute quantitative RT-PCR detection method for ACLSV in China.

ACKNOWLEDGEMENTS

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