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Simultaneous detection of five apple viruses by RT-PCR

Abstract. Apple market takes third place in the world after bananas and grapes. Apple trees are affected by viruses and virus-like diseases, which cause significant economic losses. The most common viruses in the apple tree are Apple chlorotic leafspot virus (ACLSV), Apple stem pitting virus (ASPV), and Apple stem grooving virus (ASGV). Tomato ringspot virus (ToRSV) and apple mosaic virus (ApMV) also cause significant economic losses. Timely detection of viruses and using virus-free planting materials will reduce crop losses. In this work, we performed genetic analysis of ACLSV, ASGV, ASPV, ApMV, ToRSV, and developed RT-PCR test-system for simultaneous detection of these viruses in apple tree materials. The sequences of the developed primers for the test-system are patented. The planting materials imported from Turkey and Italy were investigated for the presence of apple viruses. The most common virus in the samples is ACLSV (60%) followed by ASPV (34%), ASGV (30%) and ApMV (2%). ToRSV virus was not detected at all. Analysis of samples showed that 60% of planting materials were infected with at least one virus, some samples were infected with 3 viruses simultaneously. Mandatory certification of imported planting materials will reduce crop losses of apple orchards.
Key words: apple viruses, ACLSV, ApMV, ToRSV, ASGV, ASPV, RT-PCR, primers.

Introduction

Apple market takes third place in the world after bananas and grapes. There are over 7,500 apple tree varieties worldwide. The most common apple varieties in the world are the Red Delicious, Gala, Granny Smith, Golden delicious, Lady, Baldwin, McIntosh, Honey crisp, Fuji, and Cortland. World apple production has increased by 37.5% since 2000 and reached 59.1 million tons in 2017. The growth of the production was achieved by increasing yield, while the total planting area decreased by 10% during this time [1]. Apple export from Europe was 36.9% ($2.8 billion) of the global export. The largest exporters of apples in the world are China (US$1.3 billion or 17% of total apple export), United States ($1 billion or 13.3%), Italy ($814.1 million or 10.7%), Chile ($703.5 million or 9.2%), France ($573.4 million, or 7.5%) [2]. In 2019, apple imports to Kazakhstan reached 101 thousand tons, which amounted to 31.5% of the total consumption [3].

Apple trees are affected by at least 12 viruses and virus-like diseases, which cause significant economic losses [4-6]. Most apple viruses are transmitted through grafting. ToRSV is an exception, and it causes apple union necrosis and decline. Some apple varieties are susceptible to certain viruses. For example, it is known that all varieties of apples are susceptible to ApMV, but Golden Delicious, Jonathan, and Granny Smith are very susceptible [7]. According to various estimates, an infection can lead to loss of up to 40% of the yield, depending on the apple cultivar. ToRSV is distributed by nematodes, *Xiphinema americanum*. In the case of latent infection, viruses spread in apple trees without causing symptoms. Latent infection can lead to delaying bud blooming and decreasing in leaves size. The growth of shoots is also reduced. Further development of the disease can cause severe tree damage or death [8].

The most common viruses in the apple tree are ACLSV, ASPV, and ASGV. These viruses can be present individually or together and can cause disease in other fruit crops. Apple tree infected by these viruses shows decreasing in bud survival ability from 20% to 67%. The degree of tree damage depends on the pathogen and its strain in combination with apple

cultivar, the supply of nutrients, and the age of the tree, rhizome [9-12].

Viral control is mainly based on the prevention of possible infection by planting healthy materials and eradication of infected plants. Therefore, reliable and effective methods for early detection of viruses are important for the successful propagation of healthy plants. Within the framework of the existing programs for the agro-industrial complex development of the Republic of Kazakhstan, it was decided to create an effective system for controlling viral infections.

Apple production traditionally remains one of the main sectors of agribusiness in the South Kazakhstan region. The investigation of local strains of apple tree viruses is important since the South Kazakhstan region is located in the foothills of the Western Tien Shan, which is the natural habitat for wild apple trees *Malus sieversii* and *Malus niedzwetzkyana*. Identification of orchards affected by the virus will also provide objective data on the spreading of viruses in the country. Timely detection of viruses and using virus-free planting materials will reduce crop losses. In this work, we performed genetic analysis of ACLSV, ASGV, ASPV, ApMV, ToRSV, and developed RT-PCR test-system for simultaneous detection of these viruses in apple tree materials.

Materials and methods

Genetic analysis of five apple viruses. Analysis of genomic and gene sequences of apple viruses was performed by using Ugene V.34 programm (Russia). Genomic and gene sequences were downloaded from NCBI, S1. Multiple alignments were performed using the Muscle algorithm [13].

Development of primers for detection of apple viruses. Forward and reverse specific primers for every virus were developed by using NCBI (Primer-BLAST module) and Ugene program. The specific primers did not overlap with other nucleic acid sequences available in the NCBI database, nt-Blast. All primers were analyzed by OligoAnalyzer Tool (Merck KGaA, Germany) for their properties (GC content, Melting temperature (T_m), Secondary structure, Self-dimer & heterodimer tendencies), and compatibility in term of simultaneous detection of five viruses in a single tube.

RNA isolation. 50 samples of apple were analyzed in this work: 10 samples – Jerome (Italy), 10 samples – Pinova (Italy), 10 samples – Jerome (Turkey), 10 samples – Golden Delicious (Turkey), 10 samples – Gala (Turkey). Leaves from symptomatic trees were collected during the period of mid-April to mid-May 2019. RNA isolation from leaves was performed according to a methodology with modifications. [14]. In details, 100 mg of leaves were homogenized by grinding in a porcelain mortar with the addition of 1 ml of extraction buffer (0.1M Tris-HCl; 25mM EDTA; 2M NaCl; 2% CTAB; 2% PVP). 400 µl of homogenate were incubated at 65 °C for 10 min. After incubation, an equal volume of chloroform was added to the sample with the following centrifugation at 15,500 g for 10 min. The supernatant was transferred to a clean tube. RNA was precipitated by ethanol. The dried precipitate was dissolved in 40 µl of water.

Multiplex RT-PCR analysis. RT-PCR (reverse transcription – PCR) method was used for the simultaneous detection of five viruses. Reverse transcription was performed with a specific reverse primer for each virus (Table 1). Reverse transcription was carried out in 2 steps. Step 1 – incubation with primers: 13 µl of the reaction mixture (1 µl (150 ng) of total RNA, 1 µl (10 mM) of the specific reverse primer for each gene, 11 µl of water) was incubated for 10 min at 65 °C. Further, the reaction mixture was cooled on ice for 5 minutes. Step 2 – reverse transcription: 4 µl of 5 × RT buffer (250 mM Tris-HCl (pH 8.3 at 25 °C), 250 mM KCl, 20 mM $MgCl_2$, 50 mM DTT), 2 µl dNTP (10 mM dNTP mixture) and 1 µL (200U) reverse transcriptase (RevertAid H Minus Reverse Transcriptase) were added to the 13 µl of reaction mixture obtained in Step 1. The final reaction mixture was incubated at 42 °C for 1.5 h. Subsequently, cDNA was used for PCR with specific forward and reverse primers.

To amplify specific genomic regions of five viruses, the reaction mixture contained: 4 µL DNA from reverse transcription (cDNA), five pairs of primers at a concentration of 0.25 mM, PCR buffer, dNTP at a concentration of 0.2 mM, deionized sterile water, 1.25 U of Taq-DNA polymerase. The amplification mixture was 25 µL. PCR program for amplification: Stage 1: 1 cycle – 2 min at 94 °C; Stage 2 (35 cycles): step 1 – 20 sec at 94 °C; step 2 – 20 sec at 60 °C; step 2 min at 72 °C. Stage 3: 1 cycle – 10 min at 72 °C. All reagents used for RT and PCR were purchased from Thermo Fisher Scientific.

Results and discussion

Genetic analysis of ACLSV, ASGV, ASPV, ApMV, and ToRSV was performed to develop a highly sensitive system for detecting these viruses simultaneously in one tube. We investigated genomic regions

of viruses and selected perspective ones for primer design. These regions are highly conservative in every virus genome and do not overlap with other nucleic acid sequences available in the NCBI database.

All isolates containing complete genome of ACLSV were obtained from NCBI database.

The number of isolates was 39. The max hamming dissimilarity in 29-30 % was showed for Ta Tao 5 regarding other isolates. The min hamming dissimilarity in 8%, 5%, 0% was indicated for SY01 and SY02, BR-Gala3 and 38/85-A, QD-13 and Shanxi_14, respectively. The results of the genetic analysis of 39 genomes were used to select conservative regions for the design of forward and reverse primers. Selected regions were located in 3'- region of ORF1 encoding RNA-depended RNA-polymerase, Figure 1.

Specific primers for the capsid protein gene were used to detect ApMV [15]. Complete ApMV capsid protein genes of 29 isolates were obtained from NCBI to design specific primers, Figure 2.

Genetic analysis showed that AM490197.2 isolate of ApMV had an insertion mutation of 58 bp fragment at 3'- terminus before stop codon of capsid protein. This isolate was originated from China, isolation source – Yunnan Dounan.

All complete RNA 2 genomic sequences encoding capsid protein of ToRSV were obtained from NCBI. The number of isolates was 8. Conservative regions of RNA 2 were used to design forward and reverse primers for virus detection, figure 3. 5'- and 3'- termini are highly variable in the ToRSV genomic RNA 2.

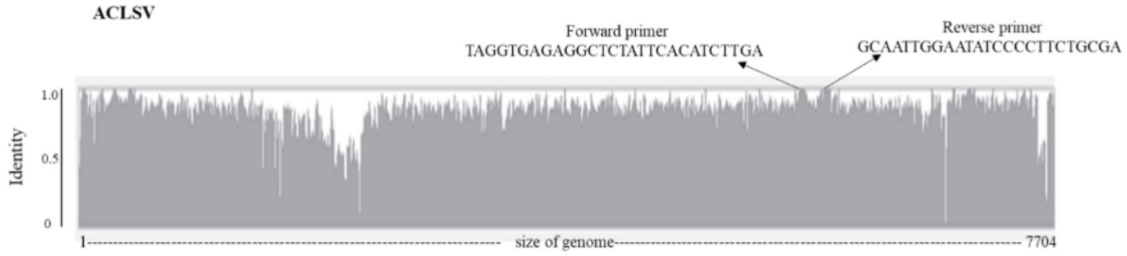

Figure 1 – Identity diagram of 39 ACLSV genomes from NCBI and primer sequences for virus detection

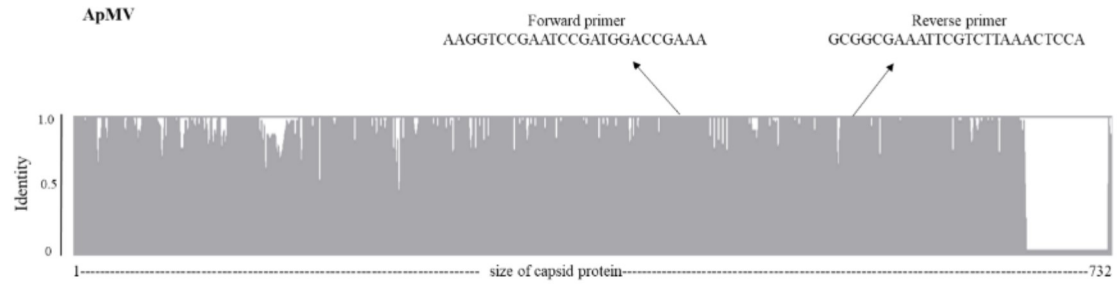

Figure 2 – Identity diagram of 29 ApMV capsid protein genes from NCBI and primer sequences for virus detection

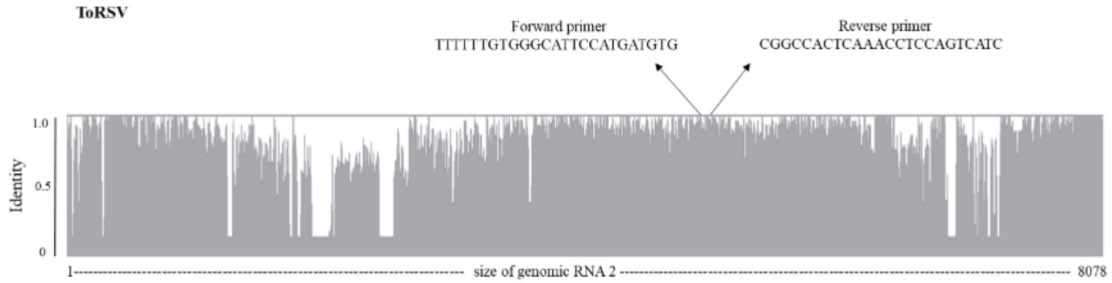

Figure 3 – Identity diagram of 8 ToRSV genomic RNA 2 sequences from NCBI and primer sequences for virus detection

ToRSV virus had multiple insertions and deletions in genomic RNA2. The largest insertion of 150 bp fragment was observed in GYV-2014 isolate.

Also, the capsid protein genes were used to design specific primers for ASGV. 130 complete capsid protein genes from NCBI were used for genetic analysis followed by primer design, Figure 4. Conservative and variable regions are shown on Figure 4.

Specific primers for ASPV were designed by genetic analysis of complete genomes of 25 isolates from NCBI. Conservative regions of RNA-depended RNA-polymerase were used for primer design, Figure 5.

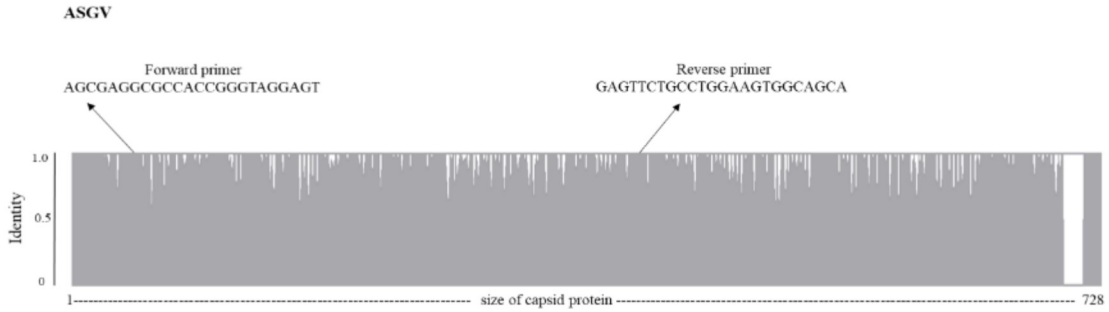

Figure 4 – Identity diagram of 130 ASGV capsid protein genes from NCBI and primer sequences for virus detection

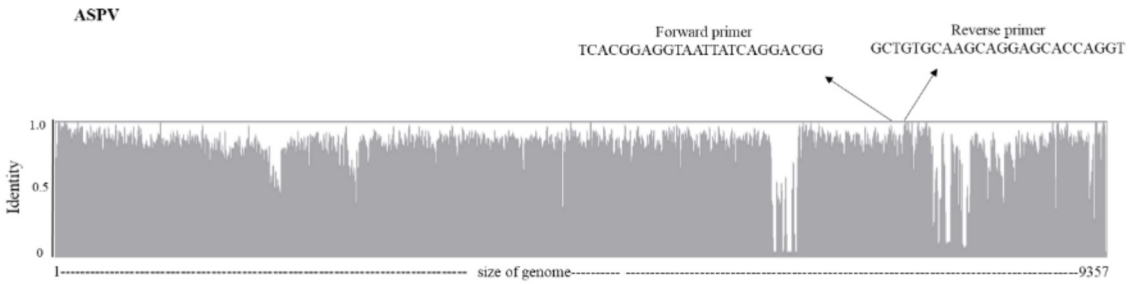

Figure 5 – Identity diagram of 25 ASPV complete genome sequences from NCBI and primer sequences for virus detection

The results of the genetic analysis revealed that the 3'-terminus of the genome encoding the triple gene protein and the capsid protein has higher variability than the 5'-terminus encoding the RNA-dependent RNA-polymerase.

The developed multiplex detection system, including specific primers for each virus, was tested on positive and negative controls. The sizes of PCR products for each virus were ACLSV – 203 bp, ApMV – 142 bp, ToRSV – 914 bp, ASGV – 378 bp, ASPV – 166 bp.

In the work, the planting materials imported from Turkey and Italy were investigated. These planting materials did not have certificates to confirm the absence of viral infection. 36 of the 50 investigated samples were infected with viruses, Figure 6.

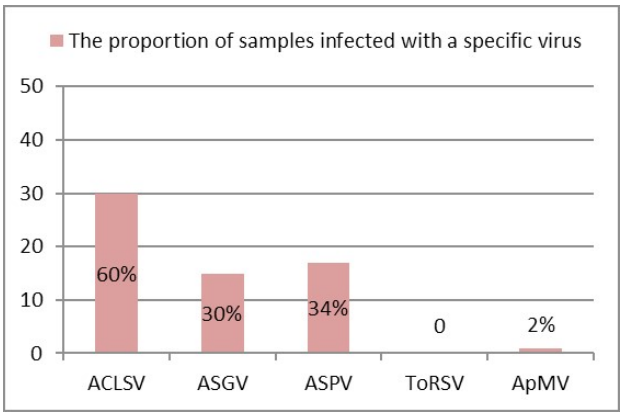

Figure 6 – Presence of five apple viruses in the planting materials from Turkey and Italy

6 samples were infected simultaneously with 3 viruses (ASGV, ACLSV, ASPV), 6 samples with 2 viruses (ASGV, ACLSV), 1 sample with ASGV and ASPV, 1 sample with ASGV and ApMV. The remaining samples were infected with only one virus. The figure shows that the most common virus in the samples is ACLSV (60%) followed by ASPV (34%), ASGV (30%), and ApMV (2%). ToRSV virus was not detected at all. All 10 Golden Delicious, 5 Jeromin, and 5 Gala planting materials from Turkey were infected with ACLSV.

Conclusion

Virus evolution is based on successful strategies that allow these intracellular parasites to multiply by utilizing the molecular mechanisms of host cells. A lot of methods have been developed to detect plant viruses, such as microscopical observation, serological techniques, molecular methods [16].

Molecular methods are used to detect viruses with high efficiency. These methods are more often used in the laboratory due to their high accuracy and sensitivity compared to serological methods. Molecular methods include PCR, PCR combined with reverse transcription (RT-PCR), multiplex PCR, isothermal amplification, microarray (oligonucleotide array).

Numerous PCR variants (multiplex PCR, nested PCR, real-time PCR) are used for virus detection. RT-PCR is very popular as a relatively cheap and reliable method for detecting plant viruses.

We performed a genetic analysis of five apple viruses and developed highly specific primers for multiplex RT-PCR test-system. Conservative and variable regions of the viral genomes were also identified by genetic analysis. Conservative regions of the viral genomes were selected for the design of species-specific primers. The primers for ACLSV and ASPV detection are specific to RNA-dependent RNA polymerase genes. Primers for ToRSV, ASGV, ASPV detection are specific to conserved regions located in the capsid protein genes. The developed primers had high specificity without formation of non-specific binding. The sequences of the developed primers are patented and can be used to detect viruses in planting materials. Controlling the possible contamination of planting materials with viral infections should be a priority in the framework of import and export programs. In this work, apple planting materials imported from Turkey and Italy were investigated by using the developed test-system. Analysis of samples showed that 60% of planting materials were infected with, at least, one virus, some samples had 3 viruses, simultaneously. Viral pathogens from infected imported planting materials can be potentially aggressive to local apple varieties. Mandatory certification of imported planting materials will reduce this threat and prevent a decrease in the yield of apple orchards.

Acknowledgments

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