First Description of Apple Rubbery Wood Virus 2 in Hungary by Small RNA High Throughput Sequencing

Az alma fapuhulás vírus 2 hazai jelenlétének kimutatása kis RNS-ek nagy-áteresztőképességű szekvenálásával

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Abstract: Apple (*Malus domestica*) is one of the most widely produced and economically important fruits in temperate regions worldwide. Recently novel negative-stranded RNA viruses have been identified in fruit trees, including pome fruit trees. Apple rubbery wood virus 2 (ARWV2) is one of them belonging to family Phenuiviridae in the order Bunyavirales. ARWV2 possess negative-sense single-stranded tripartite RNA genome small (S), medium (M) and large (L) segments. Apple rubbery wood infectious disease is characterized by unusual flexibility of twigs and smaller branches due to lack of rigidity caused by decreased lignification of xylem vessels and fibers in the symptomatic trees. In our work we detected and validated the presence ARWV2 first time in Hungary in asymptomatic apple trees via small RNA high-throughput sequencing (HTS), and detected its presence in different apple, pear and quince trees by a reverse-transcription PCR (RT-PCR) based survey.

***Keywords**: apple rubbery wood virus 2; small RNA; HTS; RT-PCR*

Összefoglalás: Az alma (*Malus domestica*) a világ mérsékelt égövi régióinak egyik legszélesebb körben termesztett és gazdaságilag legfontosabb gyümölcse. A közelmúltban új, negatív szálú RNS-vírusokat azonosítottak gyümölcsfákban, köztük almatermésűekben. Az apple rubbery wood virus 2 (ARWV2) az egyik ilyen vírus, amely a Bunyavirales rendbe a Phenuiviridae családba tartozik. Az ARWV2, egyszálú, negatív olvasatú háromosztatú RNS genommal rendelkezik kis (S), közepes (M) és nagy (L) szegmensekkel. Az alma fapuhulás fertőző betegségét a gallyak és kisebb ágak merevségének hiánya, szokatlan rugalmassága jellemezi, ami a xilém elemek csökkent lignifikációja miatt következik be. Munkánk során Magyarországon először mutattuk ki kis RNS nagy áteresztőképességű szekvenálással (HTS) a ARWV2 jelenlétét tünetmentes almafában és reverz transzkripciós PCR (RT-PCR)-n alapuló virológiai felmérésben, különböző alma, körte- és birsfák vizsgálatakor.

***Kulcsszavak**: alma fapuhulás vírus 2 (ARWV2); kis RNS; HTS; RT-PCR*

1. Introduction

Apple is an economically important fruit crop cultivated in different parts of world on large hectares of land. Apple trees are propagated vegetatively through grafting, cuttings or layering, which facilitates frequent transmission and accumulation of viruses. Apple rubbery wood virus

2 (ARWV-2)(Rott et al., 2018) is a member of the genus Rubodvirus and family Phenuiviridae. (Kuhn et al., 2020). It was first identified in apples in an association with apple rubbery wood disease (ARWD), which is characterized by unusual flexibility of stems and branches, reduced growth, shortened internodes, and increased cold sensitivity. The flexibility (rubber-like feature) of the branches in susceptible apple varieties is caused by the decreased lignification of xylem vessels and fibers in the infected symptomatic trees. ARWD was first reported in 1935 in England on apple later was found on quince and pear fruit trees. Apple rubbery wood virus 1 (ARWV-1) and ARWV-2 were discovered in apple trees together showing rubbery wood disease symptoms in Germany and USA (Jakovljevic et al., 2017; Rott et al., 2018). ARWV-2 has a tripartite negative-sense single-stranded RNA genome. The complementary RNA (cRNA) of each genomic RNA contains one open reading frame (ORF). RNAs1–3 (also named: large (L), medium (M), and small (S)) encode an RNA-dependent RNA polymerase (RdRp), a movement protein (MP), and a nucleocapsid protein (NP), respectively. Some isolates of ARWV2 have two distinct M and S RNA segments, referred as ARWV2 Ma, Mb, Sa, and Sb (Wang et al., 2022). HTS technologies are potential universal diagnostic methods which can be used to detect and identify any known or unknown pathogens present in the investigated sample (Hou et al., 2020). In our previous work we used HTS to survey the presence of pathogens in apple trees (Várallyay et al., 2022). In that work we focused on the description of citrus concave gum-associated virus, apple luteovirus 1 and apple hammerhead viroid. Detailed bioinformatic analysis of that samples indicated the presence of ARWV2 in Freedom cultivar, which was analyzed in details and validated in this current study. The introduction should briefly place the study in a broad context and highlight why it is important.

2. Materials and Methods

Apple leaf samples were collected from production orchards and germplasm collections from different geographical locations of Hungary. These samples were collected from four different branches of the trees and were used for RNA extraction by the CTAB method. For sRNA HTS, the RNA pools representing individual trees or a mixture of ten different trees were prepared by mixing equal amounts of RNA. The sRNA sequencing libraries were prepared from the purified small RNAs using a TruSeq Small RNA Library Preparation Kit (Illumina, San Diego, CA, USA) and our in-house modified protocol (Czotter et al., 2018). These small RNA libraries were sequenced using a single index on a HiScanSQ by UD-Genomed (Debrecen, Hungary) (50-bp, single-end sequencing). The sequenced reads were analysed using Qiagen CLC Genomic workbench. The reads were trimmed, both redundant and non-redundant list of sequences were prepared. This later one was used for contig building. Virus diagnostics were done by BLAST search of assembled contigs using all plant hosted viruses in the NCBI. The result list was ordered according to their lowest E-value. Both redundant and non-redundant reads were mapped to the ARWV2 reference genome. Based on this analysis the consensus sequences were prepared and the coverage of the viral genome by small RNA reads were calculated. Validation of the bioinformatic analysis to test the presence of ARWV2 was done using RT-PCR. RNA extracts were reverse-transcribed by a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher) using random primers according to the manufacturer's instructions. The quality of the cDNA was tested by amplifying a part of the *Malus domestica* actin gene. RT-PCR was carried out using cDNA as template and virus specific diagnostic primers for different segments of ARWV2. The PCR products were evaluated by gel electrophoresis. PCR products intended for Sanger sequencing were purified from the agarose

gel using a GeneJET Gel Extraction Kit (Thermo Fisher). The products were cloned into the CloneJET vector (Thermo Fisher) and Sanger-sequenced.

3. Results and Discussion

To investigate the virome of apple orchards and apple germplasm collections small RNA HTS was applied as an unbiased diagnostic method. Bioinformatics analysis of different libraries indicated the presence of ARWV2 originated contig in one library, which was prepared from a Freedom cultivar grown at Újfehertó originating from a production orchard. Coverage of the ARWV2 viral genome reached the threshold level in further samples, but although in most of them we could validate the presence of the virus using diagnostic primers designed to amplify part of the S segment, only in case of Freedom we could amplify part of the M and L segments. As ARWV2 was detected at different geographical locations we designed an RT-PCR survey to obtain further insight of its distribution and presence. Beside apple trees we sampled pear and quince at collected in different years. Out of total 77 samples15 tested positive for ARWV2 by RT-PCR using the ARWV2 310F & 824R diagnostic primers amplifying partial S segment (Table 1).

Table 1. Summary of ARWV2 positive samples by RT-PCR for S segment

Sample Origin	Position (row, tree)	Variety	ARWV2_S segment
Érd_Elvira _new certified stock2020	B VII. 41/9	Granny Smith* (apple)	+
Érd_Elvira _2019	8_13	Ozark gold (apple)	+
	8_14	Ozark gold (apple)	+
	16_3	Jonica (apple)	+
Olcsvaapáti_2020	18_4	Reglindis(E1) (apple)	+
Érd_Elvira _old certified stock_2019	1_5;1_7	Nyári fontos (apple)	+
	6_3;6_4	Akane (apple)	+
	10_2;10_3	Jonatán M41 (apple)	+
Érd_Elvira _new certified stock2021	40_28	Golden Del. Reinders (apple)	+
	37_35	Bereczki (quince)	+
	38_1	Packhams Triumph (pear)	+
	38_46	Tongre (pear)	+
	39_2	Vilmos (pear)	+
	41_1	Piros vilmos (pear)	+
		BA-29 (Rootstock)	+

While samples collected in 2019 and 2020 were confide to apples, in 2021 pears, quince and BA-29 rootstock trees were also sampled. Out of the 15 samples one apple, one quince, four pears and the rootstock were also tested positive (Figure 1).

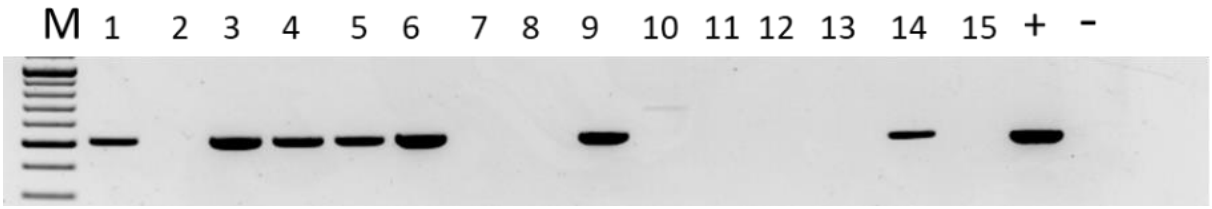

Figure 1. *RT-PCR analysis for testing the presence of ARWV2_Sa segment positive samples from location Érd_Elvira _KTÜ_2021(M-Molecular maker 100 base pair plus, 1- Golden Del. Reinders (apple), 3- Bereczki (quince), 4- Packhams Triumph (pear), 5-Tongre (pear), 6- Vilmos (pear), 9- Piros vilmos (pear), 14- BA-29 (Rootstock) and + positive control (Freedom) and -MQ H2O*

The phylogenetic tree indicates that the samples originated from Hungary clustered together, irrespectively to the host (Figure 2).

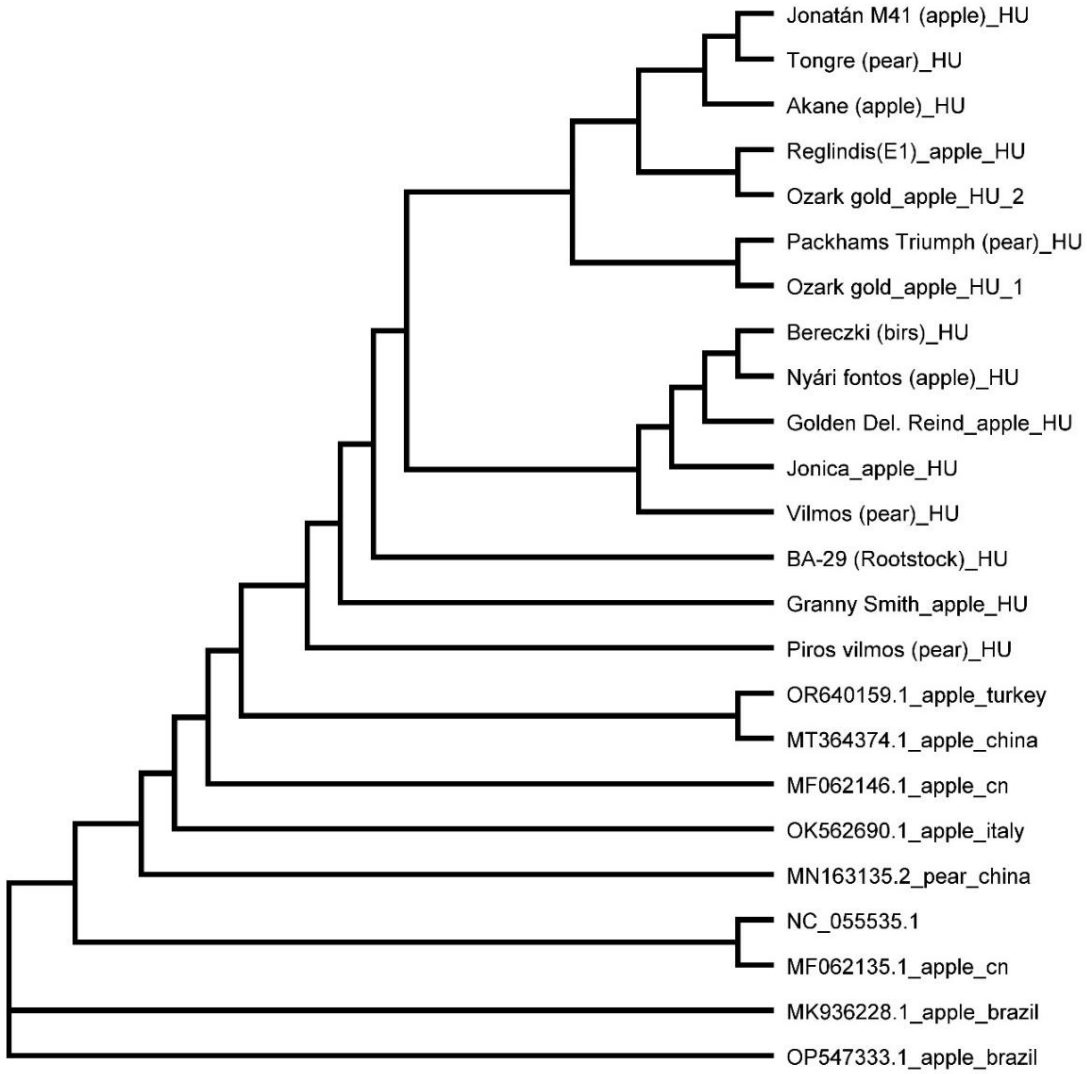

Figure 2. *Phylogenetic tree of all the Hungarian positive samples and including all the available sequences in NCBI for ARWV2 Sa segments (Geneious prime software was used to make the tree using substitution model-TN93 and 1000 bootstrap value) (HU-Hungary, CN-Canada and NC_055535.1-NCBI Id of reference genome of ARWV2_S segment*

4. Discussion

Our study provides new data and knowledge in the following areas:
1. ARWV2 was first described in Hungary using HTS and presence of all three genomic segments were validated in case of Freedom cultivar.
2. The presence of ARWV2 showed its further presence in several different apple cultivars originating from production orchards and germplasm collections from different geographical locations of Hungary and also in pear and quince.
3. Insight into the variability of ARWV2.

During our survey we detected the presence of ARWV2 in asymptomatic trees, usually infected with other viruses. To reveal the importance and possible connection between ARWV2 infection and ARWD further research is currently ongoing focusing on the changes in the miRNA regulation of the lignification process possibly induced by the ARWV2 infection.

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