Diseases Caused by Viruses

First Report of Apple Hammerhead Viroid Infecting Apple Cultivars in India

S. U. Nabi and V. K. Baranwal[†]

Advanced Center for Plant Virology, Division of Plant Pathology, ICAR-Indian Agricultural Research Institute, New Delhi

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Apple (*Malus domestica*) is widely cultivated and an important cash crop in India. Viruses and viroids have been found associated with apple in India, limiting production and productivity (Sajad et al. 2018). Of three important viroids worldwide, only apple scar skin viroid has been recorded in India (Kumar et al. 2014). In May 2019, leaf samples were collected from two symptomatic cultivars, Golden Delicious (GD) and Oregon Spur (OS), showing mosaic and ringspot symptoms and from one asymptomatic cultivar, Fuji Aztec (FA), from the field gene bank of the ICAR-Central Institute of Temperate Horticulture at Srinagar in Jammu and Kashmir Union territory, India. The samples were subjected to virome analysis using next-generation sequencing (NGS) at Nucleome Biotech (India). Total RNA of each sample was extracted using a QIAGEN RNeasy Plant minikit as per the instructions, and each sample was quantified and quality checked by an Agilent 2100 Bioanalyzer, NanoDrop (Thermo Fisher Scientific), and 1% agarose gel. Total RNA (1 µg) with RNA integrity number >7 was used for library preparation. The NGS library was prepared as per the manufacturer's protocol (NEBNext Ultra RNA Library Prep Kit for Illumina, New England Biolabs) and sequenced on a HiSeq 2500 sequencer. The sequence data, approximately 40.75, 32.35, and 27.15 million of 100 nt raw reads (from OS, GD, and FA) were run through a classification tool, Kaiju (v. 1.6.3) using the NCBI BLAST *nr* +euk database as a reference to identify sequences of nonhost origin. Contigs were generated by de novo assembly using Trinity (v. 2.3.0) (Haas et al. 2013) from the remaining 55,831, 57,381, and 14,312 reads, respectively. Assembled contigs underwent BLASTn

analysis against the NCBI nucleotide, viroid, and virus databases. Results revealed, apart from viruses (apple necrotic mosaic virus, apple mosaic virus, and apple stem grooving virus), leaf samples from two trees of OS and GD were also infected with apple hammerhead viroid (AHVd), which was first reported in China (Zhang et al. 2014) and recently from the USA, Japan, Italy, Spain, and New Zealand (Szostek et al. 2018). From 55,831 and 57,381 reads of samples OS and GD, 10 and 17 contigs were formed, respectively. A single contig of 436 nt from OS and 434 nt from GD could cover the entire AHVd genome; none of the contigs from FA matched AHVd. AHVd-OS and AHVd-GD isolates shared 99.51 and 93.35% sequence similarity with AHVd reference sequences from New Zealand and Italy, respectively (MH649334, MH643720). AHVd in GD and OS was confirmed by one-step RT-PCR using the primers AHVd-1F/AHVd-1R and amplifying the entire genome of AHVd (Messmer et al. 2017). The specific amplicon of 440 bp obtained was cloned in pGEM-T Easy vector (Promega) and sequenced, confirming AHVd infection. The sequences of both isolates were deposited in GenBank: MN786529 (AHVd-GD) and MN786530 (AHVd-OS). Subsequently, leaf samples from a single tree of six commercial apple cultivars showing mosaic and necrosis (OS, Red Delicious [RD], GD, Royal Delicious [RoD], Gala Mast, and Red Gold) were screened for AHVd using RT-PCR, as described. The AHVd-specific amplicons of 440 bp were obtained from OS, RD, GD, and RoD only; the other two tested negative. The amplicons from OS and RD were cloned and sequenced. The sequences of both showed 98 to 99% nucleotide identity with AHVd isolate (MH649334). So far, detection of AHVd was possible only from apple, signifying a single host species (Serra et al. 2018). Previously, AHVd was detected in apple trees showing trunk splitting (Messmer et al. 2017), shoot decline, and dieback symptoms (Szostek et al. 2018). In this study, the viroid was found associated with trees exhibiting mosaic and necrosis symptoms from four cultivars. However, its role in symptom expression is not yet clear, because other viruses were also found in these symptomatic trees. This is the first report of AHVd from the apple cultivars in India. This study indicates expanded distribution of the viroid in Asia.

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[†]Indicates the corresponding author.

E-mail: vbaranwal2001@yahoo.com (V.K.B.)