Variability assessment and construction of infectious clone of Indian *Apple Scar Skin Viroid*

Walia, Y.; Kumar, Y.; Rana, T.; Ram, R.; Hallan, V.; Zaidi, A.A.
Plant Virology Lab, Institute of Himalayan Bioresource Technology, CSIR, Palampur, 176061, Himachal Pradesh, India.

Abstract

Apple scar skin viroid (ASSVd) is widely distributed and economically important pome-fruit infecting viroid belonging to the genus *Apscaviroid*. It causes huge economic losses to the apple industry. Apple fruits with dappling, scarring, cracking and deformation symptoms were noticed during survey of apple growing regions of Himachal Pradesh, India. ASSVd was detected from four isolates showing dappled fruits. Molecular characterization of the viroid was done. Ten clones each from five isolates were sequenced out of which seven new sequence variants of ASSVd were found. Four of the clones were 330 nucleotides (nt) long and the other eight had an additional nucleotide. The clones showed significant sequence variability (94-100%) with each other. Variability was more common in the pathogenic domain of the viroid genome. Present isolates grouped with some Chinese and Korean isolates in phylogenetic analysis. The study reports seven new sequence variants of ASSVd and also gives a first molecular evidence of a viroid infection (ASSVd) in apple from India. Infectious clone of ASSVd were constructed for *in vitro* mutagenic studies.

Keywords: *Apple scar skin viroid*, cloning, DNA sequencing, phylogenetic analysis

Introduction

Viroid genome consists of single stranded covalently close circular, non-coding, non-encapsidated RNA. Without encoding capacity, the viroid RNA genome and its replication intermediates interact directly with host components for nearly all aspects of infection process, including replication, intercellular movement, intercellular movement, systemic movement and pathogenecity. They exist as rod-shaped structures in their secondary state (Diener, 2001). Viroids have been grouped into two families, *Pospoviroidae* (nucleus replicating) and *Avsunviroidae* (chloroplast replicating) based on their mode of replication (Flores et al., 2005). ASSVd is the type species of genus *Apscaviroid*, family *Pospiviroidae* (Hashimoto et al., 1987).

Apple is one of the major fruit crops of India and cultivated in the northern states (Himachal Pradesh, Jammu and Kashmir, Uttarakhand) and some north-eastern states (Arunachal Pradesh, Sikkim and Nagaland). Apple can be infected with viroids viz. ASSVd, *Apple fruit crinkle viroid* (AFCVd) and *Apple Dimple fruit viroid* (ADFVd) (Hashimoto and Koganezawa, 1982; Di Serio et al., 2002; Koganezawa, 2001).

These viroids cause severe symptoms on apple fruits which include color dappling, cracking, scarring and distortion depending upon the cultivar (Koganezawa, 2001) rendering it totally unmarketable. Three other diseases, dapple apple (Hadidi et al., 1990), pear rusty skin (Chen et al., 1987) and Japanese pear fruit dimple (Osaki et al., 1996) have been attributed to ASSVd molecular variants (Zhu et al., 1995). In India, this viroid has already been reported based on symptomatology and PAGE assays (Handa et al., 1998). In the present communication ASSVd is detected and molecularly characterized from apple bark tissue from Northern India. Apple plants were found to be infected with complex mixture of sequence variants of ASSVd. To the best of our knowledge this is the first molecular report of a viroid infection in apple from India. Infectious clones of the ASSVd were constructed to study infectivity and host pathogen interactions.

Materials and methods

Molecular characterization of ASSVd: Surveys were conducted in different apple growing regions of Himachal Pradesh. Forty- five fruit and bark samples of the symptomatic apple plants were collected and analyzed for the presence of ASSVd. Total RNA was extracted using RNeasy® Plant Mini kit (Qiagen, Germany) with slight modification in the extraction buffer. From the total RNA, RT-PCR was performed on standard conditions using ASSVd specific primer pair (cASSVD and hASSVD; Di Serio et al., 2002). For first strand cDNA synthesis, a reaction mixture of 25µL was made using 7µL RNA, 5µL M-MLV reverse transcription buffer (5x, USB), 1µL (200ng/µL) primer (down primer), 1.5µL dNTP mix (40mM), 0.5µL (200 U/µL) M-MLV reverse transcriptase (USB) and 0.1µL (40U/µL) RNase inhibitor. The RT reaction was incubated at 37 ºC for 75 min and then at 70 ºC for 5 min. PCR was carried out in 0.2ml thin walled tubes in an automated thermocycler (Applied Biosystems, USA). PCR reaction mixture

consisted of 10µL of cDNA, 5µL 10x Taq DNA polymerase buffer (Genei, India), 2µL of dNTP mix (10mM), 1µL (200ng/µL) each of downstream and upstream primers and 0.5µL Taq DNA polymerase (Genei, India).

PCR products were run on 1 % agarose gel, stained with ethidium bromide (1µg/ml) and visualized under UV transilluminator. PCR products were purified from the gel using GenElute Gel Extraction Kit (Sigma, USA) and the eluted products were cloned into pGEM®-T Easy vector (Promega, USA). Recombinant plasmids were purified using GenElute Plasmid Miniprep Kit (Sigma, USA) and sequenced with an automated DNA sequencer (ABI PRISM® 3130xl Genetic Analyzer) using ABI prism Big Dye™ Terminator v3.1 Ready Reaction Cycle Sequencing Kit (Applied Biosystems), sequencing both strands. Sequences were submitted to EMBL database and accession numbers AM993159, AM993160, FM178283, FM178284, FM178285, FM208138, FM208139, FM208140, FM208141, FM208142, FN547406 and FN547407 were obtained. Sequences were analyzed with the help of Basic local alignment search tool (BLAST; Altschul et al., 1990). Multiple alignments were carried out with the help of MultAlin software available online at

http://bioinfo.genopole-toulouse.prd.fr/multalin/multalin.html (Corpet, 1988).

For phylogenetic analysis the accession numbers of different ASSVd sequences taken from GeneBank are: EU031496, EU031489, AF421195, X17696, EU031464, DQ362906, M36646, GQ249350, GQ249349 and FJ974063. An isolate of *Apple dimple fruit viroid* (EF088665) reported from Italy, was taken as an out-group. Origin of these isolates has been shown in the phylogenetic tree. The modified ClustalW program (Chenna et al., 2003) was used (www.ddbj.nig.ac.jp) with 1000 bootstrap replicates and phylogenetic tree was viewed using TreeExplorer software (Kumar et al., 1994) downloaded from the site http://www.megasoftware.net/

Construction of Infectious Clones: Infectious ASSVd clones were constructed by dimerizing of the ASSVd sequence. Primers were designed from the unique *Sal*I restriction site region of ASSVd sequence at nucleotide position 87. Homologous primer with the *Sal*I site (87-106 nts, ASSVdICh; GTCGACGAAGGCCGGTGAGAA), and complementary primer (87-67 nt, ASSVdICc; CGTCGACGACGACAGGTGAGTT) was used for amplifying the viroid and then the same homologous primer in combination with complementary primer without the *Sal*I restriction site (81-62 nt, ASSVdIC1; GACGACAGGGTGAGTTCCTTC) was used for genome amplification. Both of the amplified products were cloned into pGEM®-T Easy vector (Promega, USA) and sequenced. Recombinant plasmid containing the insert amplified by primer pair ASSVdICh and ASSVdC1 were digested with *Sal*I restriction enzyme, purified using Phenol: Chloroform: Isoamyl alcohal (25:24:1) solution and ligated using T4 DNA Ligase (Fermentas). The ligated product was transformed into *E.coli* DH5α strain. Recombinant plasmids were purified using GenElute Plasmid Miniprep Kit (Sigma, USA) and sequenced. Finally, plasmid containing the dimer sequence was cloned into binary vector pCambia-1300 and transformed into agro bacterium GV3101 strain.

Results and discussion

Expected sized amplicons of ~330 bp were obtained in five isolates from three apple varieties viz. Royal delicious (four isolates), Gold Spur (one isolate) and Red Chief (one isolate). Ten randomly selected cDNA clones were sequenced from each positive sample and identified as ASSVd in BLAST search. The sequences showed 94-100 % similarity with each other. Seven clones were found to be new sequence variants of ASSVd. Two clones (AM993159 and AM993160) showed 100% similarity to a Chinese isolate (EU031496) whereas eight clones (FM178283, FM178284, FM208139, FM208140, FM208141, FM208142, FN547406 and FN547407) showed 99% similarity to a Korean isolate (AF421195). Remaining two clones (FM178285 and FM208138) were more similar to the Chinese (EU031455) and Japanese isolate (M36646). The Korean isolate is 331 nt long and differs from the Japanese isolates in its 'G' insertion between the nucleotide 133-134 (Lee et al., 2001).

However all sequences reported in this communication had the 'G' insertion, though four of them are 330 nt and other eight are 331 nt long. Eight 331 nt long ASSVd clones contain a 'T' insertion between the 220-221 nt which lacks in the other four ASSVd clones. Sequences of the present clones were aligned with the ASSVd sequences reported from other locations (Figure 1). Variability was identified at 25 positions out of 310 (excluding primer sequences), which were more common in the 5'-end of the putative pathogenicity domain. This suggested that ASSVd is quite variable in its sequence in this domain, which might be responsible for different symptom expressions in different plants.

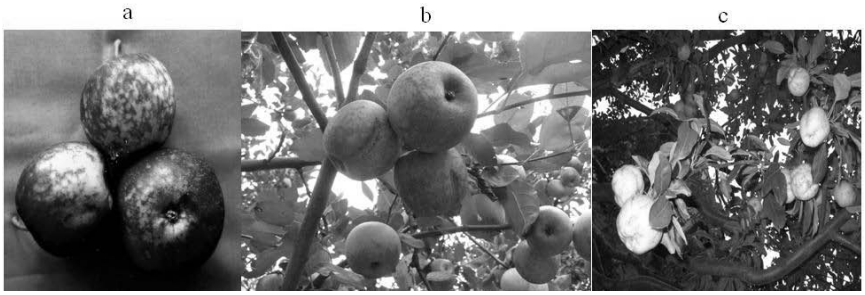

Fig. 1 a) Dappling symptoms on apple fruits, b) Scarring of apple fruit skin c) Deformation of the apple fruit.

In the phylogenetic analysis (Figure 2) our clones grouped with Chinese, Japanese, Korean isolates, which suggests that these have a geographical relationship, as all these countries are part of Asian continent and possibly there could be an exchange of material among them. Interestingly, AM993159 and AM993160 clustered with a Chinese isolate (EU031496) reported from apricot, which suggests their close relationship.

Greece isolates from cherry, wild apple and pear also showed close resemblance with our isolates. All other isolates clustered with different apple isolates reported from China, Korea and Japan (Figure 3).

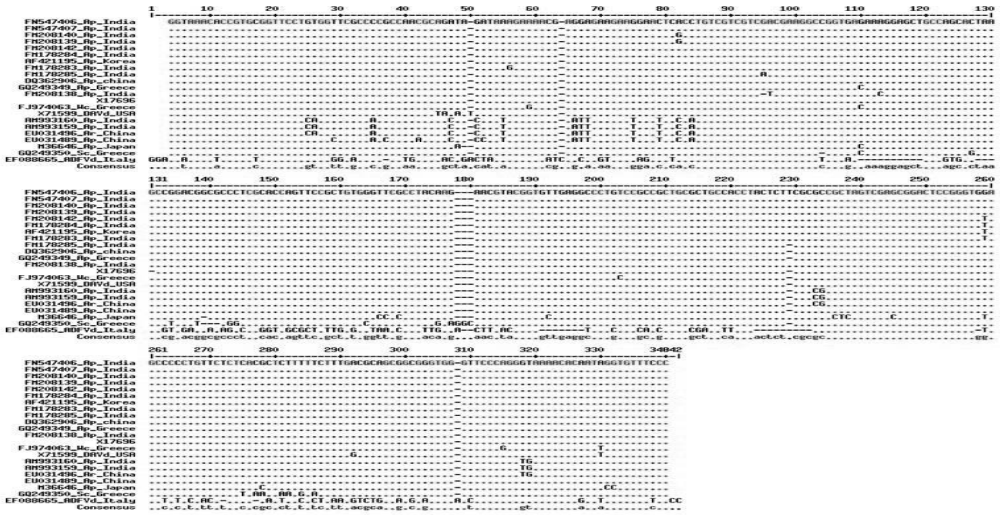

Fig. 2 Multiple alignment of the present isolates showing variability in first 90 nucleotide part and the central conserved region.

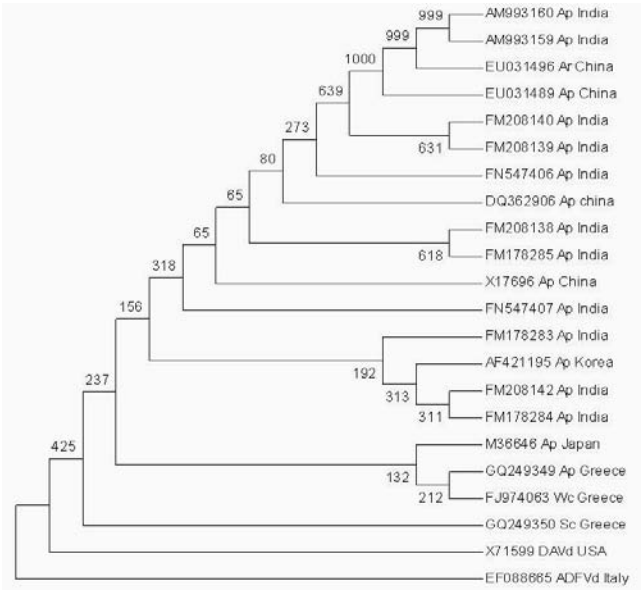

Fig. 3 Phylogenetic tree showing evolutionary relationship of the present isolates with other ASSVd isolates taken for study. AM993159 and AM993160 show close relationship with some Chinese isolates reported from apricot. Other clones cluster with different apple isolates reported from China, Korea, Greece and Japan. Numbers at the nodes of the branches indicate bootstrap values out of 1000 replicates (In percent). *Apple dimple fruit viroid* sequence (EF088665) reported from Italy, was taken as an out-group. Ap: Apple; Ar: Apricot; wc: Wild Cherry; wa: Wild Apple.

The dimer of ASSVd was constructed successfully and experiments involving the agroinoculation of these constructs into different hosts are under process. All five domains of the viroids of family *Pospoviroidae* can cause symptom alterations in the host. Mutational analysis of *Potato spindle tuber viroid* and construction of intra-specific chimeras have shown that sequences within at least three of these domains (i.e. the left terminal loop, pathogenecity domain and variable domain/right terminal loop) play important roles in modulating symptom expression (Visvader et al., 1986; Sano et al., 1992) and mutations in the loop E of the CCR of the *Pospoviroids* suggested that CCR can also play role in the symptom development (Qi and Ding, 2003). ASSVd causes symptoms of dappling (new apple cultivars) as well as scarring (old apple cultivars). Symptoms depend upon the cultivars (Desvignes et al., 1999) and probably on the viroid variants infecting the cultivars. Thus mutational studies with ASSVd may help us to predict which particular nucleotides could be responsible for the specific symptoms on apple fruit. The study will make a base for viroid research of temperate fruits in India. To the best of our knowledge this is the first molecular evidence of viroid infection on apple in India.

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