

Elucidating fate of *Apple scar skin viroid* in animal cells

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Abstract: *Apple scar skin viroid* (ASSVd) has been identified in nine different herbaceous hosts and also reported to be actively transmitted by the whitefly *Trialeurodes vaporariorum* (Tv). Not only the viroid RNA, its DNA form was also identified from the insect. We report here the host range of ASSVd which was further explored in the mammalian system to elicit the infection of the viroid in the animal CHOK-1 cells. For this, CHOK-1 cells were transfected with ASSVd transcripts *in-vitro* and screened for its replication potential. Confirmation of the presence of the viroid replicative forms was also done. The integration of the viroid in the genomic DNA of CHOK-1 cells was also worked out. The replicative forms of the viroid were characterized and analyzed bioinformatically. This finding is highly relevant in the present scenario as now apple is being cultivated in lower hills where whitefly vector, viroid, herbaceous hosts and humans are in close association. There is an urgent need to understand the fate of this pathogenic RNA in animal cell lines, which forms the basis of this study.

1 Introduction

Viroids are the smallest known infectious pathogens. They are solely composed of a short strand of circular, single-stranded RNA without protein coat. Their genome, consists of a non-coding, circular RNA of size ranging from 246-401 nucleotides (nt). It is autonomously replicated by host-encoded proteins. Based upon their structure and region of replication in the plant cell the viroids are divided into two families the Avsunviroidae and the Pospiviroidae. The Pospiviroidae members have rod shaped secondary structure and replicate in the nucleus by asymmetric rolling circle amplification method. The members of the family Avsunviroidae have branched shaped secondary structure in which hammerhead ribozyme is embedded and they replicate in the chloroplast via symmetric rolling circle amplification method.¹ Viroids are transmitted via vegetative propagation/grafting and mechanical inoculation, e.g., through the use of contaminated blades during pruning and grafting. It has been observed that the PSTV gets encapsidated by the coat protein of Potato leaf roll virus and gets transmitted by *Myzus persicae* and Tomato chlorotic dwarf viroid could be transmitted by bumble bees during pollination.^{2,3} ASSVd naked RNA was found to be directly transmitted by the whitefly and no virus was found associated with the transmission of ASSVd.⁴

Transfection is a powerful analytical tool enabling study of the function of genes and gene products in cells. Using

various chemical, lipid or physical methods, this gene transfer technology is a powerful tool to study gene function and protein expression in the context of a cell. The introduced genetic materials (DNAs and RNAs) exist in cells either stably or transiently depending on the nature of the genetic materials. The entire "CHO cell system" encompasses a variety of different cell lines which were likely all derived from a clonal and spontaneously immortalized Chinese hamster ovary cell.⁵ CHO cells represent the most frequently used mammalian production host for therapeutic proteins due to several key advantages over other cell types such as: (i) a robust growth in chemically defined and serum-free suspension culture, (ii) a reasonable safety profile regarding human pathogenic virus replication, and (iii) the ability to express r-proteins with human-like post-translational modifications.^{6,7} Apple is a perennial plant and therefore the losses caused to growers due to ASSVd infection are substantial. Since a large part of the population is dependent on the apple industry for consumption and post-harvest products which require good quality and disease free fruits, hence it is necessary to determine the impact of the viroid in human and animal cell lines. Thus, we report the following: (i) Replication potential of ASSVd RNA in transfected cell lines; and (ii) Identification of the viroid DNA form (if any)

2 Results and Discussion

ASSVd dimer, cloned in TA cloning vector (RBC, Taiwan) glycerol stock was revived from -80°C on LA ampicillin positive plate by streaking with an incubation at 37°C for overnight. In vitro ASSVd transcripts were prepared from the cloned viroid genome containing plasmid, linearized with BamHI enzyme was then transcribed by in vitro transcription reaction using T7 RNA polymerase enzyme.

CHOK-1 animal cell lines which were transfected with "Attractene" (QIAGEN) transfection reagent at 60-80% cell confluency for different time points. Cells were thawed from liquid nitrogen vapour phase. After the cells have attained their exact morphology and have complete adherence to the substratum of the culture dish, these were trypsinized and seeded after the first passage. With respective negative controls and samples at different time points namely 6 hrs, 12 hrs, 24 hrs, 48 hrs and 72 hrs, about 4 µg of viroid transcripts were transfected in 100mm culture dish with 2-2.5 million cells in each dish and suitable culture media.

Cells were incubated for about 12-16 hrs, with the transfection media and monitored for suitable growth environment. Meanwhile, if the culture media was found to be depleted then the media was replenished under aseptic conditions.

The normal healthy CHOK-1 cells present an adherent epithelial-like morphology as shown in Figure 1(A). Transfection experiment was setup with respective negative controls and subsequently after incubation with transfection complexes, media was changed and fresh C-Hams growth media was added followed by RNA isolation at different time points. Post transfection, significant changes were observed in the cell morphology and density in accordance with the time point of RNA isolation.

The negative control groups comprised of the RNA from healthy CHOK-1 cells alongwith RNA from healthy CHOK-1 cells with ASSVd transcripts and without transfection reagent "Attractene" in which the morphology was unaffected without any significant changes as shown in Figure 1(B). The third control group was the mock control in which the healthy CHOK-1 cells were treated with attractene reagent without viroid transcripts as observed in Figure 1(C) in order to check the effect of the stress induced which resulted in altered morphology and deposition of debris on the cellular substratum. In treatment groups, evident changes in cellular morphology

as well as cell viability were seen. In Figure 1(D), at 6 hrs of RNA isolation, no evident change alongwith stable growth of cells was observed. Cellular properties viz., viability, growth and morphology were maintained at 12 hrs of RNA isolation showing features of stable transfections as seen in Figure 1(E). At the next three time points of cell harvest, cells started to lose their intrinsic morphology i.e, epithelial-like and attained more striated form with signs of cell death like globularization of cells, cell shrinkage, plasma membrane blebbing, cell detachment leading to necrosis and cell death as observed in Figure 1(F,G and H)

RNA was isolated from animal cells using QIAGEN RNeasy extraction kit (Hilden, Germany). The quality and integrity of the extracted RNA was confirmed by electrophoresis in 1.5 per cent agarose gel. RNA isolated from CHOK-1 cells was checked for ASSVd infection by RT-PCR using viroid specific ICc (Reverse) and ICh (Forward) primers, which amplified a 330 bp fragment. The amplified products were eluted and confirmed as ASSVd by sequencing

From the intensity of the band of the amplicon in Figure 2[A], it can also be inferred that the forms of the viroid are present in large quantity from 6 hrs to 24 hrs time point and gradually decreases between 48 to 72 hrs. The sequencing of the eluted RT-PCR product showed 100 per cent homology to Indian isolate of Apple scar skin viroid isolate C3 (NCBI BLASTN). We analyzed monomeric circular and linear RNAs of the viroid for confirming its presence in the transfected CHOK-1 cells. RNA preparations were fractionated on a denaturing formaldehyde gel however, the RNA reproducibly separates into two distinct bands upon gel electrophoresis in formaldehyde also observed in case of PSTV⁸. The finding by electron microscopy of covalently closed circular and linear RNA molecules in preparations of denatured purified PSTV suggested that the two bands consist of the circular and linear forms of PSTV, respectively^{9,10}. Analysis of the denaturing gels via northern blot hybridization with a viroid-specific riboprobe indicated the presence of both the monomeric circular and monomeric linear forms (Fig. 2 B) in transfected CHOK-1 cells, whereas no bands were observed in the control groups. The viroid replicates in the mammalian system especially at 12 hrs and 24 hrs time point the Monomeric

circular and Monomeric linear forms are quite evident and intense.

The area under the band determines the band intensity that can be correlated to the quantity of the viroid RNA forms present in the transfected cells at different time points. It is observed from the graph that the titre of replicative forms increases gradually from 6 hrs and attains its maximum at 12 hrs. The titre then decreases from 24 to 72 hrs, abruptly.

A similar kind of study was released lately where Mycoviruses of an endophytic fungus can replicate in plant protoplasts of two different plant hosts, *N. benthamiana* and *N. tabacum* BY2 cell line¹¹. Since, the presence of the viroid forms was confirmed from the northern blot, identification of the DNA form was also done. To explore the identity of this DNA form, rolling circle amplification (RCA) was performed using transfected and control CHOK-1 cells. Circular DNA was only amplified using samples from transfected CHOK-1 cells as observed in figure 4[A]. Total RCA products amplified from transfection and control samples at different time points were digested using BamHI (Thermo Scientific, USA), DraII (Fermentas, USA), Sall (Takara Bio Inc. Japan), and HincII (Thermo scientific, USA) restriction enzymes by overnight digestion. RCA product from all the transfection samples couldn't be digested by using these restriction enzymes in figure 4[B] which indicates that the amount of free forms available is too low to be amplified by RCA alone. Predictably, a part of it is integrating in the genomic DNA, which needs to be investigated further.

Total DNA of the transfected cells was isolated and tested for a possible DNA form of the viroid using PCR. Transfected CHOK-1 cells yielded positive amplification signals, which were confirmed by sequencing and Southern hybridization, using viroid-specific primers (ASSVdICc and ASSVdICb), whereas healthy CHOK-1 control cells tested negative. The PCR results showed amplicons of various sizes alongwith the dimeric and monomeric DNA forms of the viroid figure 5[A]. Non-specific amplifications were observed which may be due to different concatameric forms of the circular DNA.

The dimeric viroid form of size 600 bp was found to hybridize with ASSVd specific probe in Southern blotting. The Monomeric form of the viroid was not observed to hybridize with the probe in the blot indicating non-specific amplifications in the PCR.

On contrary to the northern blot, maximum amplification was clearly observed as intense bands at 6 hrs in the southern blot (Lane 1& 2, Fig 5 B) indicating large amounts of viroid form integrated between 6 hrs to 12 hrs time point.

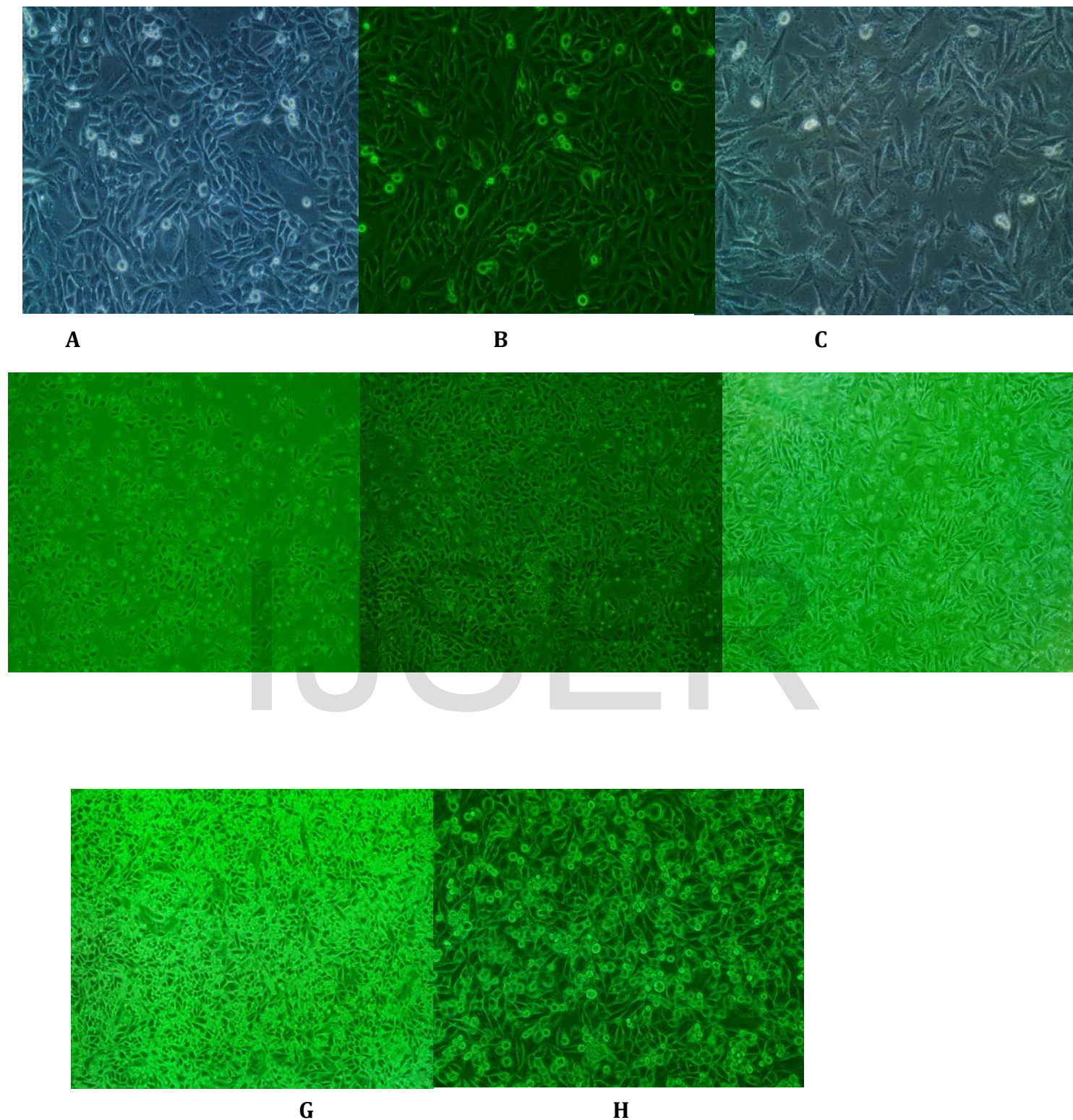
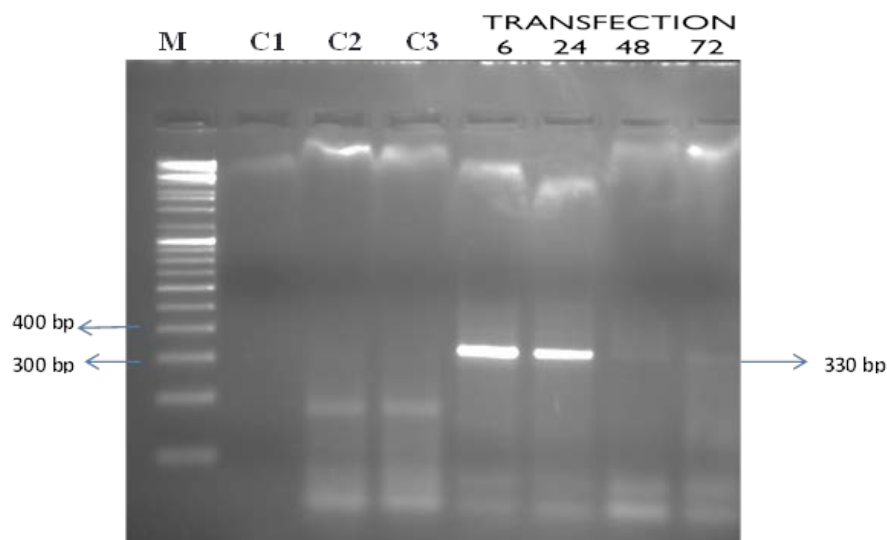
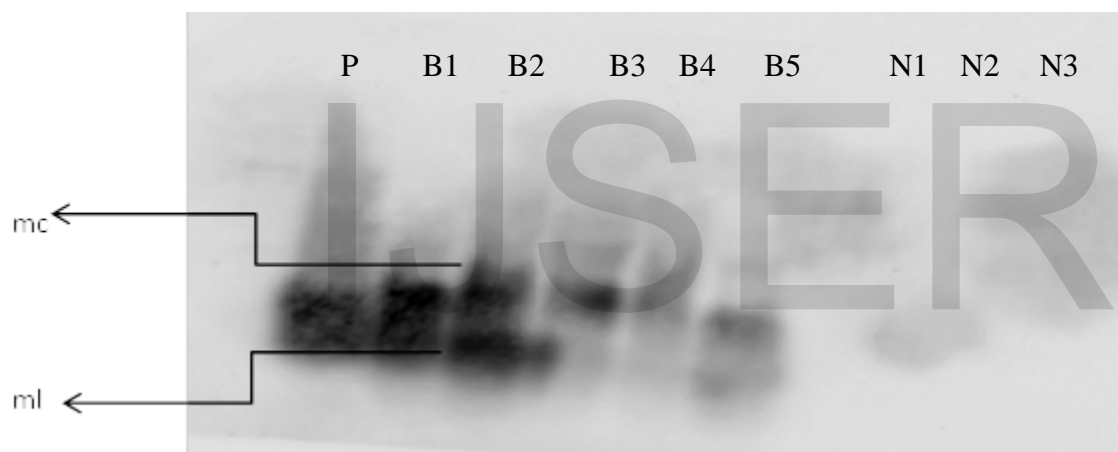


Figure 1: Cells observed in Phase Contrast Microscope at 20X magnification



A



B

Figure 2: A: RT-PCR results and B: Northern Blot results. P Positive Control ; B1- CHOK-1 cells transfected with dimeric transcripts (6hrs); B2- CHOK-1 cells transfected with dimeric transcripts (12hrs); B3 -CHOK-1 cells transfected with dimeric transcripts (24hrs); B4- CHOK-1 cells transfected with dimeric transcripts (48hrs); B5 -CHOK-1 cells transfected with dimeric transcripts (72hrs); N1- Negative control (healthy cells); N2- Negative control (healthy cells + viroid); N3- Mock Control ; mc: monomeric circular & ml : monomeric linear.

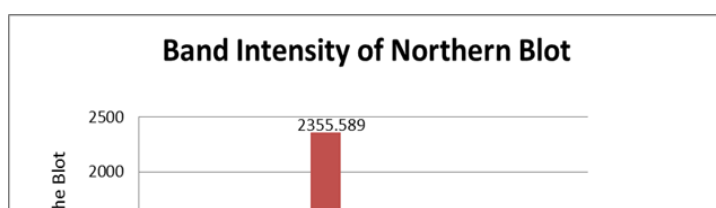


Figure 3: Graph showing relative replication of the viroid RNA forms with respect to different time points from the band intensity in the northern blot.

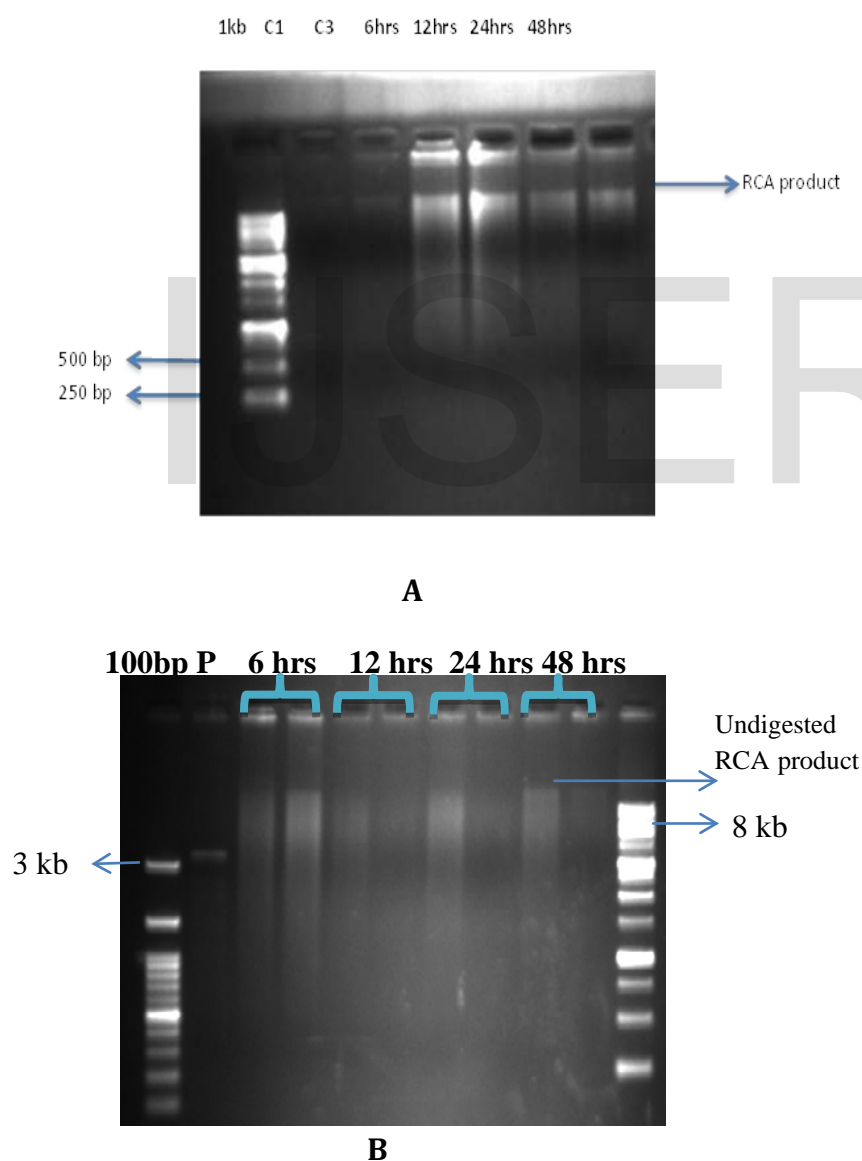


Figure 4: A- Agarose gel electrophoresis analysis of the RCA product. C1 and C3: control DNA from healthy cells and mock control, respectively with RCA product of transfection samples at different time points and B- Agarose gel electrophoresis

of overnight digestion of the RCA product with *SalI* and *HincII*. P: Positive control ASSVd dimeric plasmid cloned in TA with undigested RCA product of transfection samples at different time points.

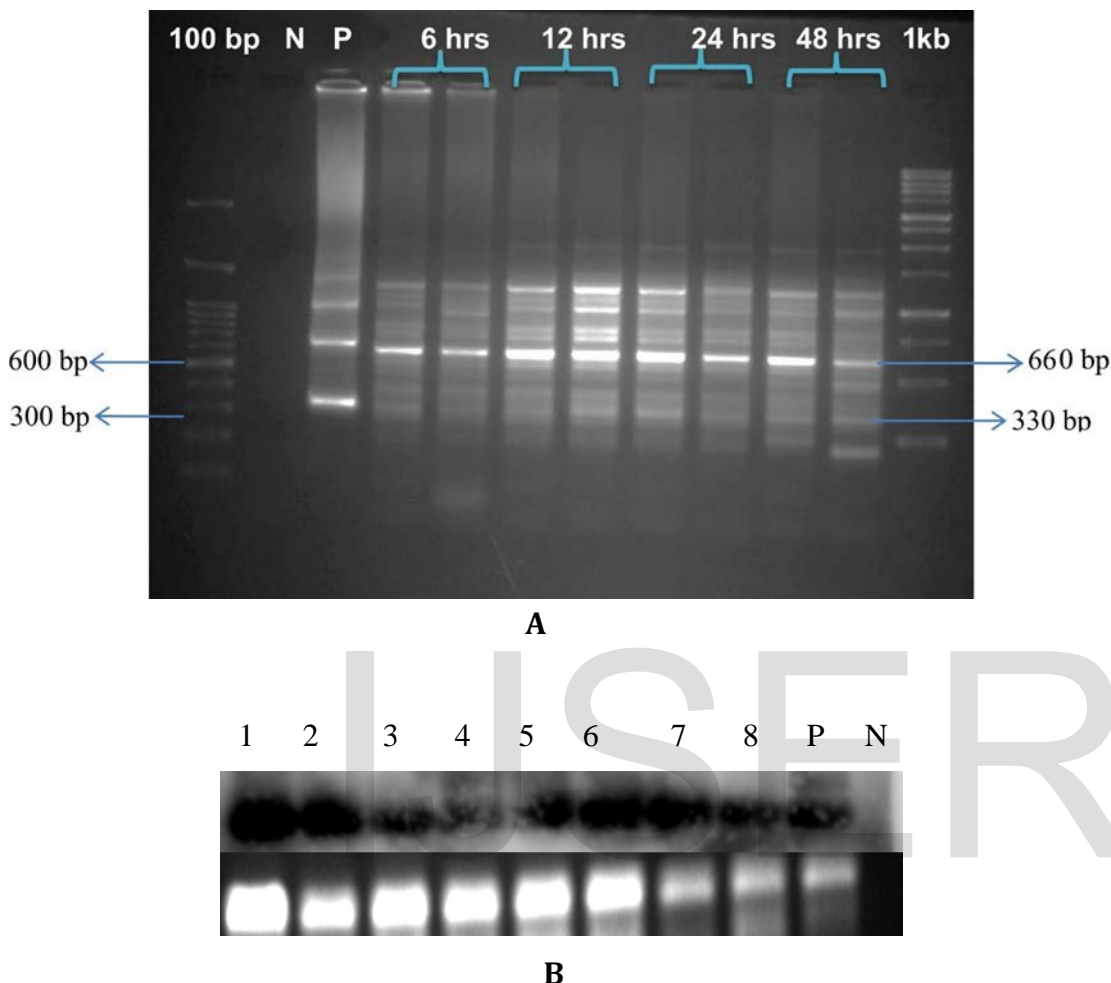


Figure 5: A- Agarose gel electrophoresis of amplified PCR product from RCA product. N: Negative control (C3), P: Positive control (ASSVd dimeric plasmid cloned in TA) followed by transfected samples at different time points. B- Southern blot analysis: N: Negative control (C3- Mock control), P: Positive control (ASSVd dimeric plasmid), RCA product of transfection samples 1 & 2: 6 hrs, 3 & 4: 12 hrs, 5 & 6: 24 hrs, 7 & 8: 48 hrs.

3 Materials and Methods

Generation of viroid transcripts

ASSVd dimer cloned in TA cloning vector (RBC, Taiwan) stored in DMSO at -80°C was revived on a Luria agar plate containing ampicillin by streaking method and grown overnight at 37°C. Individual colonies were taken from overnight incubated Luria agar plate and sub cultured in 5 ml Luria broth containing ampicillin for selection incubated for about 12-16 hrs at 37°C and 225 rpm in an incubator

shaker for pure plasmid isolation. 1 µg of ASSVd Pure Plasmid was linearized by *Bam*HI enzyme followed by Phenol Chloroform Isomyl Alcohol (25:24:1) purification. 1µg of PCI purified linearized plasmid was used for in-vitro transcription as per Fermentas kit recommendations. Transcribed RNA was checked by agarose gel electrophoresis.

Cell culturing of CHOK-1 cell line

CHOK-1 (ATCC® CCL-61™) cell line having origin from the ovary of Chinese hamster exhibiting an epithelial-like adherent morphology was procured from National Centre for Cell Science (NCCS), Pune, India that is a non-profit organization providing high quality established cell cultures that are carefully tested to ensure the authenticity of the cells. Cryovials with frozen cells were procured from liquid nitrogen container or -80°C refrigerator. The culture medium (HAMS) used for their growth was supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS; Life Technologies, USA) and 1% (v/v) antibiotic-antimycotic (Life Technologies, USA). Cells were cultured under aseptic conditions and passaged once a week after trypsinization (using 0.25% trypsin containing 0.2% EDTA) and residual medium was replaced with fresh medium twice a week. Cultures were maintained in CO₂ incubator (Thermo Scientific, USA) with 95% humidity, 5% CO₂ at 37°C. After 2nd passage cells were used for Transfection after trypsinization¹².

Transfection of viroid transcripts to CHOK-1 cells

Cells were trypsinized and seeded in 100mm culture dishes as 3 for control groups and 5 for treatment groups, a day before transfection as per requirement. Counting of cells is done by haemocytometer to ensure optimal cell density [i.e., Cells should be 40–80% confluent on the day of transfection] and physiological conditions at transfection. The cells were incubated under normal growth conditions (typically 37°C and 5% CO₂). On the day of transfection, 4 µg RNA was dissolved in TE buffer, pH 7–8 (minimum RNA concentration: 0.1 µg/µl) with medium without serum, proteins, or antibiotics to a total volume of 60 µl as per culture dish. 15 µl Attractene Transfection Reagent was added to the RNA solution. Mixed by pipetting up and down or vortexing. Samples were incubated for 10–15 min at room temperature (15–25°C) to allow complex formation. The transfection complexes are added drop-wise onto the cells. The plate was gently swirled to ensure uniform distribution of the transfection complexes. The cells were incubated under their normal growth conditions and analyze the cells after an appropriate time, if cytotoxicity is observed, remove the complexes after 6–18 hours and add fresh culture medium.

RNA isolation of Transfected cells

RNA was isolated at different time intervals after the removal of transfection complexes at 6, 12, 24, 48 and 72 hours. The cells were harvested from the culture dish by adding 1000µl RLT (Lysis) Buffer with 10µl β-mercaptoethanol followed by scrapping off the cells. RNA was extracted from the cell lysate by QIAGEN RNeasy kit and checked on 1.5% agarose gel for its integrity.

Detection of ASSVd via RT-PCR

RT-PCR was performed using total RNA under standard conditions and an ASSVd-specific primer pair (cASSVd and hASSVd). PCR was performed with one cycle at 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s, and a final extension at 72°C for 10 min. The RT-PCR products were analyzed on a 1.5% agarose gel.

Northern blotting of transfected RNA

Total RNA (10µg) of the ASSVd transfected RNA was separated by denaturing 1% formaldehyde agarose gel prepared by dissolving 0.9g of the agarose in 88ml of 1X MOPS buffer [0.1M MOPS, 40 mM Sodium acetate, 5 mM EDTA, adjust pH to 7 with NaOH]. The solution was heated in a microwave oven to dissolve the agarose completely and then in a fume hood, 2% of 37% formaldehyde solution was added. EtBr was added and poured into the gel tray. After capillary transfer to nylon membrane (Hybond-N, Amersham Pharmacia Biotech), it was fixed with ultraviolet (UV) irradiation. The membranes were incubated at 65°C in hybridization buffer (5X SSC, 0.1% N-lauroyl sarcosine, 0.02% SDS, 1% blocking reagent; Roche) with DIG-labeled RNA probes specific for the positive ASSVd strand. Stringent washings were given to the blot¹³. Chemiluminescent detection was performed using CDP star (Ambion) according to the manufacturer's instructions.

Isolation of CHOK-1 Transfected DNA

Healthy and ASSVd transfected CHOK-1 cells were harvested by trypsinization and spun for 5 min at 300 x g. The pellet was suspended in 200µl PBS along with 20µl proteinase K. Total DNA from the pellet was extracted by QIAGEN DNeasy kit as per company recommendations.

RCA of CHOK-1 DNA and PCR

RCA was performed on DNA from healthy and transfected CHOK-1 cells using TempliPhi DNA polymerase (GE

healthcare) according to the manufacturer's instructions at 30°C overnight. PCR was performed as described above.

Southern Blotting of RCA-PCR

RCA-PCR products were run on 1% agarose gel and preceded for capillary transfer overnight and fixed by UV irradiation. Hybridization and Washings were done with the blot¹³. Chemiluminescent detection was done by CDP star as mentioned above.

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5 References

1. Flores R, Randles JW, Joseph BM and Diener TO .1998. A proposed scheme for viroid classification and nomenclature. Archives of Virology 143: 623-629
2. Antignus Y, Lachman O and Pearlsman M .2007. The spread of Tomato apical stunt viroid (TASVd) in greenhouse tomato crops is associated with seed transmission and bumble bee activity. Plant Disease 91: 47-50
3. Syller J and Marczewski W.2001. Potato Leafroll Virus-assisted Aphid Transmission of Potato spindle tuber viroid to Potato Leafroll Virus-resistant Potato. Journal of Phytopathology 149: 195-201
4. Walia Y, Dhir S, Zaidi AA and Hallan V. 2015. Apple scar skin viroid naked RNA is actively transmitted by the whitefly *Trialeurodes vaporariorum*. RNA Biology 12: 1-8
5. Puck TT, Cieciura SJ and Robinson A .1958. Genetics of somatic mammalian cells. III. Longterm cultivation of euploid cells from human and animal subjects. J. Exp. Med 108:945-956
6. Kim TK and Eberwine JH .2010. Mammalian cell transfection: the present and the future. Anal. Bioanal. Chem. 397:3173–3178
7. Kim YJ, Baek E, Lee JS and Lee GM .2013. Autophagy and its implication in Chinese hamster ovary cell culture. Biotechnol. Lett 35:1753–1763
8. Diener, T. O. & Smith, D. R. (1973) Virology 53,359-365.
9. McClements, W. L. (1975) Ph.D. Thesis, University of Wisconsin, Madison.
10. McClements, W. L. & Kaesberg, P. (1977) Virology 76, 477- 484.
11. Nerva L, Varese GC, Falk BW and Turina M. 2017. Mycoviruses of an endophytic fungus can replicate in plant cells: evolutionary implications. Scientific Reports 7: 1908-1919
12. Freshney R. Culture of Animal Cells: A Manual of Basic Techniques, John Wiley & Sons, 1983, New York, United States. John Wiley & Sons Inc
13. Sambrook J, Fritsch E, Maniatis T. Molecular Cloning: A Laboratory Manual, Cold Spring Harbour, 1989 New York, USA. Cold Spring Harbour Press