



Official publication of Pakistan Phytopathological Society  
**Pakistan Journal of Phytopathology**

ISSN: 1019-763X (Print), 2305-0284 (Online)

<http://www.pakps.com>



**BIOLOGICAL CONVERSION OF POTATO SPINDLE TUBER VIROID (PSTVD) RNA INTO NONINFECTIOUS ENTITY BY A SINGLE BASE SUBSTITUTION IN *NICOTIANA BENTHAMIANA* VAR DOMIN**

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**ABSTRACT**

It is matter of common understanding that when we inoculate some strain of pathogen to model plant, it moves from source to sink. The *Potato spindle tuber viroid (PSTVd)* was first identified viroid of family Pospiviroidae, was isolated from Potato leaves i.e. natural host and submitted to Genbank (Accession # MK303578). The *PSTVd* is a single stranded circular RNA having 359 nucleotides which causes mild to severe symptoms in experimental host. *Nicotiana benthamiana* was selected as model targeted host and inoculated with mutated PSTVd (at base position 171). A single base change created at base position 171 of loop motif '26' from A to U (T) has stopped PSTVd replication and make it noninfectious. The RNA was extracted from host and converted into cDNA and sequenced again. The inoculated plant with a single base change showed no infection. No infection symptoms on experimental host plant. The results of sequencing and BLAST and Mfold (version 3.0) confirmed a single base change at position 171 of Loop 26 of PSTVd. This was a unique approach to change genetic sequence of PSTVd to minimize/stop its infection and spread.

**Keywords:** *Potato spindle tuber viroid*, beta marceptaethanol, RT-PCR.

**INTRODUCTION**

*Potato Spindle Tuber Viroid (PSTVd)* is first recognized viroid belongs to family Pospiviroidae [which are considered as the subviral RNA plant pathogens in having single stranded circular RNAs, ranges from 260-450 nucleotides base pairs which cause infection to host (Bagherian *et al.*, 2014). The reproductive mechanism of viruses and viroids is same. It takes the control of the reproductive factory of host plant for the synthesis of its own RNA. It is also considered that these infectious RNAs as an original ancestor of small viral RNAs, Which have no ability to code some amino acids for protein synthesis and have to rely on hosts for its replication. These qualities differentiate them from satellite RNAs and plant viruses (Boonham *et al.*, 2004).

The movements of these RNA pathogens like PSTVd is very

*Submitted: October 17, 2018*

*Revised: December 27, 2018*

*Accepted for Publication: May 14, 2019*

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important for understanding plant physiology and development (Bostan *et al.*, 2004). The interaction of plant host and pathogen is also very important for establishing a systemic infection by viroids. Increasing evidences have proved that these kinds of processes are regulated by specific type of RNAs (Bostan *et al.*, 2004).

The PSTVd provides a unique model to understand function and structure of RNA motifs for causing an infection. The different Loops of PSTVd have been worked out before for understanding their functions. A single base substitution from C to G, A to U (T) and reversion can change pathogenic efficiency of PSTVd was first time reported by Wassenegger (Candresse *et al.*, 2010). During the last decades, several loops motifs of PSTVd have been investigated for this purpose (Constable and Moran 1996; Di Serio, 2007; Ding, 2010).

The concept of site directed mutagenesis in the PSTVd has opened new era of discussion. The inoculation of PSTVd on leaves of host plant cause possible movement from source to sink. A single base substitution can produces a new mutant which don't trafficks into host plant (Ding and Itaya 2007; Fels *et al.*, 2001). So we have focused on loop 26 at

base position 171 analysis and single change was made at position 171 (A-T) by site directed mutagenesis.

*Nicotiana benthamiana* is the model species for plant virology and contemplating plant-organism collaborations. It is additionally a model for certain plant physiological activity. We have tried to evaluate the mechanism of viroids pathogenicity using PSTVd mutant as an experimental system. In this concern we reported that a special change in at position 171 of viroidal genome can help to decrease its infection.

#### **MATERIAL AND METHODS**

**Isolation of PSTVd RNA:** The first and foremost step of our study was to isolate PSTVd from infected plants i.e potato., which were recognized on the basis of symptoms as mentioned in literature (Di Serio, 2007). The PSTVd RNA was solated from infected Plants by modified Trizol method. The prior addition of beta marceptaethanol (BME) in trizol , helped to improve fine extraction of PSTVd RNA (1µl BME/1ml Trizol) /The infected frozen plant tissues were ground in 500 µl of modified Trizol reagent. For phase separation added 100 µl of chloroform reagent and vortexed for 20 seconds then incubated at room temperature for almost 30 minutes. This sample was again centrifuged centrifuged at 13000rpm for 15 minutes. Two phases were formed i.e lower red interphase and colourless aqueous phase. The upper phases was collected in separated tubes then added 250µl of cold Isopropanol at room temperature and left for 10 minutes . After incubation period of ten minutes this mixture was again centrifuged at 13000rpm 4 °C for 20 min. The pellet was collected and 500 µl 75%ethanol was added and centrifuged again for three minutes at 750rpm (1 ml of 75 %ethanol was added as per 1 ml of trizol reagent). The pellet was dried and suspended in 500 µl of Trizol reagent. The storing pellate in Trizol for required period of time was another modification of our described method. RT-PCR was run with PSTVd specific primers (Gozmanova, 2003). The presence of PSTVd was confirmed by BLAST and Sequencing.

**Site Directed Mutagenesis:** The Loop 26 of PSTVd consisting of 8 nucleotides (ACAUUCCU). The first nucleotides was selected and Primer wwasdesigned (AAUCAGGGCCCUUCCUUU) for cDNA (AATCAGGGCCCTTCCTTT) with single base change from A to U (T) at position 171. The Reverse transcription process was used to make PSTVd mutant by the protocol (Hammond, 1992).

**Plant growth and viroidal infections:** The

*N.benthamiana* plants were grown in control chambers maintained at 16 hours light and 8 hours dark photoperiod. The inoculation of this mutant (250ng/leaf) was done on first two true leaves of host plant. Before inoculation small abrasion were also made on leaves with carborundum powder. The original wild type extracted RNA was also inoculated as positive control at the same time. The Diethyl Pyrocarbonate (DEPC) treated water was used for mock inoculation. Twenty one days of post inoculation the RNA was extracted from inoculated leaves (Postive & Negative control) .

#### **RNA Extraction with Modified Trizol Protocol**

**cDNA Synthesis:** For completion of experiment extracted RNAs were converted in to cDNA on same day. The GScript First strand synthesis Kit (Cat No MB 305-0050) was used to convert RNA into cDNA following manufacturer's instruction.. Extracted RNA (2µl) was added in eppendorf and Kit's component I.e Oligo (dT) 1 µl and dNTPs mix (1 µl) were mixed in same tubes and centrifuged for 5 min at 13000 rpm. The 13 µl of nuclease free water was added in centrifuged sample. It was heated in water bath for almost 3-5 min at 65°C. when incubation period was completed the whole mixture was spun shortly on vortex and shifted on ice briefly. 5x ist strand buffer of kit was also added as 4 µl. Gscript Rtase and DTT (0.1M) were added 1 µl Respectively (Ding, 2010).

**Agarose GEL run:** Agarose Gel was run and bands were noted and scored. The quality of extracted PSTVd cDNA was checked on a 1% agarose gels stained with Ethidium bromide and visualized under µtraviolet light. The quantity of was PSTVd RNA and cDNA also noted with spectrophotometry. All samples were found between 1-2 nm UV-range. The quantification of cDNA was also performed with <sup>TM</sup>Nanodrop 2000/2000c Thermo Fisher Scientific V1.0 The larger peaks at260/280nm were obtained for purified sample. The Visualization of bands were done in gel documentation system and was photographed. The high molecular and high quality bands were seen and scored for good quality c DNA (Hammond, 1992).

**Sequencing:** The PSTVd cDNA were submitted to BioBasic Canada inc for Sanger Dideoxy Sequencing. The obtained seq files (ID: Ist Base 3332384\_3P) were analyzed Bioedit and FASTA sequence was submitted to gene bank and got accession number i.e Accession # MK.303578. The RNA unfolding studies M fold also confirmed the base change at position 171 of viroidal genome.



Table 1. Morphological l changes observed in *N. benthamiana* infected by PSTVd mutant 21 days of post inoculation.

Sr No.	Mutation Sequence	Number of plants inoculated	Disease Symptoms	Number of Plant showed trafficking	Trafficking efficiency%	Pathogenic efficiency
1	A→U	40±0.52	No obvious symptoms	0.0±0.33	00±0.00	-
2		40±0.52	No obvious symptoms	1.0±0.33	10±0.00	-
3		40±0.52	No obvious symptoms	0.0±0.33	0.0±0.00	-

The mean with different letter in each column are significantly different according to Duncan’s multiple range test (0.005p value) ± = Standard error

It was recorded in (Table 1) that trafficking/ transport of PSTVd completely changed after base change in viroidal genome.

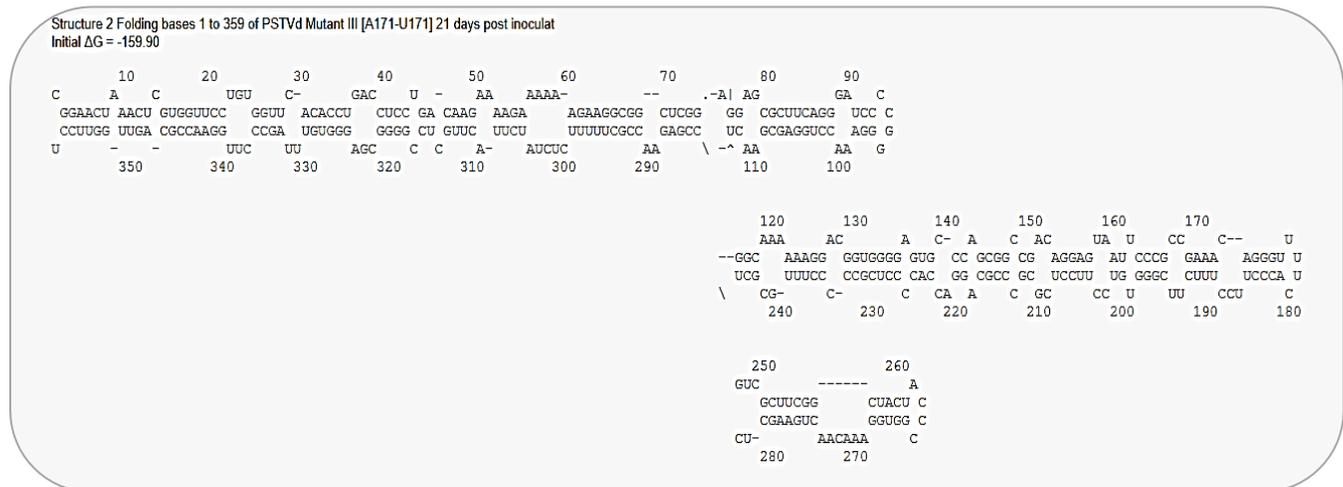


Figure 7. Fold analysis of PSTVd Mutant with Base Position number 171 A is replaced with U (A171-U171) after 21 days of inoculation to *N.benthamiana*

**DISCUSSION**

The extraction of PSTVd and trafficking has been reported as an area of genomics from last decades by many researchers in different countries (Arif *et al.*, 2005; Ding, 2010). The RNA of pathogens moves from cell to cell and cell to organ through special opening ‘plasmodesmata’ (Fels *et al.*, 2001). Increasing evidences have reported that these process are regulated by RNA movements inside the cells (Gozmanova, 2003; Guner *et al.*, 2012) The study of PSTVd RNA and its structural loops motifs has enabled us to investigate function-structure relationships for short and long distance transport. The unique feature of PSTVd make it a model to study different loops and motifs (Ding, 2010). About 11 to 14 loops have been PSTVd genomic RNAs have already been investigated in *Nicotiana benthamiana* (Hammond and Owens 1987). The nucleotides base substitution from A to U, U to G A to T has disturbed the function of loops which indirectly has effected movements of PSTVd in host

plants (Kasai *et al.*, 2013). The present research is different and first time reported in details for gain and loss of functions of PSTVd mutants. Sometime a base substitution A/C can lead to new Loop formation and function of PSTVd movements restore as happened in loop19 (Kasai *et al.*, 2013). The inoculation of PSTVd RNA has produced the symptoms of reduced growth and colour change in leaves. These symptoms have already been reported by the work of Kolonko *et al.*, 2006). The presence of viroids RNA was confirmed by sequencing of cloned products that have amplified in PCR with PSTVd specific Primers with no base changes (Kolonko *et al.*, 2006). A total of 10 to 12 microgram of PSTVd and PSTVd mutant was used for inoculation onto *Nicotiana benthamiana* plants. Three weeks later the inoculated plants leaves were harvested. The total RNA was extracted and cDNA was prepared similarly discussed by the research of Ding (Ling *et al.*, 2014). The Plants inoculated with PSTVd show symptoms but mutant inoculated plants have not shown any

symptoms and gave no bands when run on a gel. This work was in accordance with the work of other researchers of same domains Mackie *et al.*, 2015; Mahfouze, 2008). The site directed generated mutant of PSTVd have not shown any symptoms to model plant (Owens, 2007; Owens and Baumstark 2007) The sequence comparison of PSTVd FASTA with other sequence has shown a change at position 171 out of 359 nucleotides by sequencing analysis.

#### CONCLUSION

The presented study have proved as an outstanding and efficient method for site directed mutation of PSTVd RNA, its mutant generation and amplification from infected plants like Potatoes. The single base substitution of PSTVd and making it non infectious is a new addition to existing research.

**Conflict of Interests Statement:** Authors declare that there is no conflict of interest for publishing this study.

#### ACKNOWLEDGEMENT

The and Molecular genetics and Plant Biotechnology Lab in the Department of Botany, Lahore College for Women University, provided all the necessary items during present studies. Some part of the research was also executed under the Guidance of Sir M. Dr M. Ashfaq and help of hard working student Mubarak Ali Anjum in Institute of agriculture, University of Punjab. We are very thankful to kind cooperation of different students of both labs.

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