IDENTIFICATION OF POTATO SPINDLE TUBER VIROID (PSTVd) FROM DISEASED PLANTS OF TOMATO (*LYCOPERSICON ESCULENTUM* MILL.) IN PAKISTAN

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ABSTRACT

Potato Spindle tuber viroids is a low molecular weight, infectious single stranded RNA which is first time discovered in potato. This pathogen has also infected other members of Family Solanaceae. The degradation and instability of any plant RNA is main interruption for dealing with extraction and isolation of these plant pathogenic RNAs and further processing. Mostly RNAs degraded as we take tissues from plants. A very different and brief protocol for PSTVd RNA isolation and storage has been improved and optimized. The present study was aimed to discover and confirm the presence of PSTVd in infected tomatoes in Pakistan. PSTVd infected plants were collected analyzed on the basis of symptoms and signs. The total RNAs was isolated and purified using new optimized Trizol reagent. The RNA of infected tomatoes was converted to cDNA on same day and amplified with reported primers. The identification and confirmation of PSTVd was achieved by BLAST and Sequencing (Accession MK 303578.1. MK977647). The advance optimized Rapid Trizol reagent method was found best and reproducible. The extracted RNA which was stored in Trizol also gave best result even after six months. It is also proved that RNA could be store in trizol reagent for 6 months.

Key words: PSTVd, RNA, Trizol, BLAST, cDNA, Pathogenic

INTRODUCTION

Potato Spindle tuber Viroid' was the first viroid to be identified. It is a smallest infectious circular Single stranded RNA that causes infection to many ornamental plants, crops and weeds (Bostan *et al*., 2003). This pathogen is a member of Pospiviroidae family of viroids. About last four to five decades ago there was no discrimination in signs and symptoms between viruses and viroidal disease till the discovery of viroids was characterized and reported by Candresse *et al*. (2010). The genomic map of PSTVd consisting of a 359 nucleotides bp long, single-stranded RNA, which forms rod-like conformation, having five distinct domains. Viroid is considered a novel and smallest phytopathogen. Presently, about 30-35 diseases of plants have been documented as being instigated by viroids in agricultural plants horticulture and ornamental plant species all over the world (James, *et al*., 2008). Viruses and Viroids are responsible for 40-50 billion of field losses every year effecting stored and cultivated crops equally, proving themselves one of the major hurdle for effective food production and supply. These Viroids are considered as a novel class of plant pathogen at molecular level when compared to plant viruses (Nie, 2012). Viroids are also reflected as the sophisticated and most efficient 'molecular plants parasites' apparently flaking the requirement to manufacture functional/structural proteins by operating enzymes from targeted host for their own replication and are skilled of destroying even long and strong trees, of coconut and palms (Hadidi *et al*., 2003). The Short-lived vegetables and herbaceous annual crops e.g. tomato, potato, capsicum etc, which are grown by true seeds, display maximum loss by such infections (Salazar, 1995). All class of plant viroids is communicable by process of grafting but some of these are transmissible by pollen and seeds of diseased plants (Singh, 1970; Singh *et al.,* 1993). There is a tremendous increase in number of plant viroids identified in last three decades, which have the attention of global community to protect their fields and farms from exotic and imported phytopathogens (Arif *et al*., 2005). Two decades earlier, it was a general opinion that any organism without a protein coat could not be able to replicate itself, even with the help of host cell protein. However, discovery of PSTVd proved it wrong. The PSTVd was the first viroid identified, as physical entity, native to Australia and also posed serious threat to world economy (Fels *et al*., 2001). Approximately 32-33 families of plants have shown mild to severe sensitivity to viroids (Flores *et al*., 2005) The members of solanaceae were affected badly by this pathogen including tomatoes, potatoes and chili. The complicated interaction of plant pathogen and host machinery is the key behind the signs and symptoms of disease development (Verhoeven *et al*., 2010). The degree and type of infection depends of systemic transfer efficiency (source to sink). PSTVd exhibits mild to severe symptoms in Solanaceous plants. Diagnoses and identification of

PSTVd depend on symptoms, which appeared on leaves or stems of infected plants like Chlorosis retarded growth in tomatoes and purpling/redding of young leaves (Ling *et al*., 2014). Tomato is one of ten most vital vegetables and fruits used around the world. It is estimated that 134.6 million tons fresh tomatoes are farmed and harvested in the world. According FAO reports of 2016, In Pakistan, 72.5 thousand hectares of tomato gave yield of 608258 tons (FAO, 2016). This vegetable is characterized with high nutrition value. It is cultivated in tropical, sub-tropical region (Bagherian *et al*., 2014). Although a lot of work has been done on extraction of RNA from infected plants tissues, but this is first report of Isolation of PSTVd RNA from infected plants of tomatoes in Pakistan with modified methods. The Isolation of RNA is always a big problem and challenging experiment in university laboratories. It is not always conceivable to track and follow available protocols due to complicated and expensive apparatus required. The fast efficient protocol for isolation of PSTVd was optimized. This was found very brief and operative protocol completed almost three to four hours. Another advantage of this method is that extracted RNAs can be stored for longer duration. For completion of experiment some amount of required RNA was also shifted into c DNA for further down processing.

MATERIALS AND METHODS

Screening and Collection of infected plants

Plants were screened and collected on basis of symptoms reported in literature (Arif *et al*., 2005; Bagherian *et al*., 2014). The fresh leaves were analyzed with yellowing and redding of tomatoes leaves and stunted growth of complete plants (Fig.1). The leaves of infected plants were plucked and covered in Aluminum foil the whole tissues were immediately shifted to liquid nitrogen jars.

Fig 1. Infected tomato plants, leaves and stem with short sized and Chlorosis symptoms.

Isolation of PSTVd RNA

The infected frozen plant leaf tissues of tomatoes were ground in Trizol reagent (500 µL) and placed for 10 to 15 minutes on ice. (For better working of Trizol almost 2 µL of Beta marceptanol was added per ml of trizol reagent). This prior addition helped to obtain fine quality RNA from infected RNA. For clear phase separation we have added 100 µL of chloroform reagent. The complete mixture was vortexed (for 20 seconds) and incubated at room temperature for almost 30-34 minutes. This sample was centrifuged at 13000/rpm for almost 15 minutes. Two phases were appeared one was lower red interphase and second colourless aqueous phase. The upper phase in tube was collected and about 250µL of cold Isopropanol was added at room temperature for 5-10 minutes. This all mixture was again centrifuge at 13000rpm 4 ^{o}C (20 min). The clear pellet was formed and collected, 500µL 75% ethanol was added and centrifuge for three minutes at 750rpm. (1 mL of 75 % ethanol was added as per 1 mL of trizol reagent). The flow through of tube was discarded and pellet was dried and suspended in 500 µL of modified Trizol reagent (having β-marceptanol) (Sano *et al*., 2010).

cDNA Synthesis for further downstream processing

For further processing required isolated RNA was converted in to cDNA on same day. The GScript First strand synthesis Kit (Cat No MB 305-0050) was used to transform PSTVd RNA into cDNA. The protocol was maintained and optimized following manufacturer‘s Instruction. Extracted RNA solution was added in centrifuge with Oligo (dT) 1 µL and dNTPs mix 1 µL. The 13 µL of Nuclease free water was added. It was heated for almost 3-5 ^{o}C at 65 ^{o}C when incubation was completed the whole mixture was spun shortly and shifted on Ice briefly. The provided 5x ist strand buffer of kit was added 4 µL. Gscript Rtase and DTT (0.1M) was added 1 µL, respectively. The amount of enzymes and dntps were also optimized for getting fine quality DNA.PCR was also run for these samples. (Owens and Hammond, 2009)

Gel Electrophoresis of PCR samples

Gel was run for the PCR samples 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 ultra violet light. The quantity of was also noted with spectrophotometry. All samples were found between 1-2 UV-range. The Quantification of cDNA was also performed with ^{TM}Nanodrop 2000/2000c Thermo Fisher Scientific V1.0 The large 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 / (Fig. 2) (Hammond *et al.*, 1989: 2009)

Fig 2. Les *Lycopersicon esculuntem* sample 1 to 9 PSTVd RNA from infected samples gave Sharp bands of amplified PSTVd Cdna.

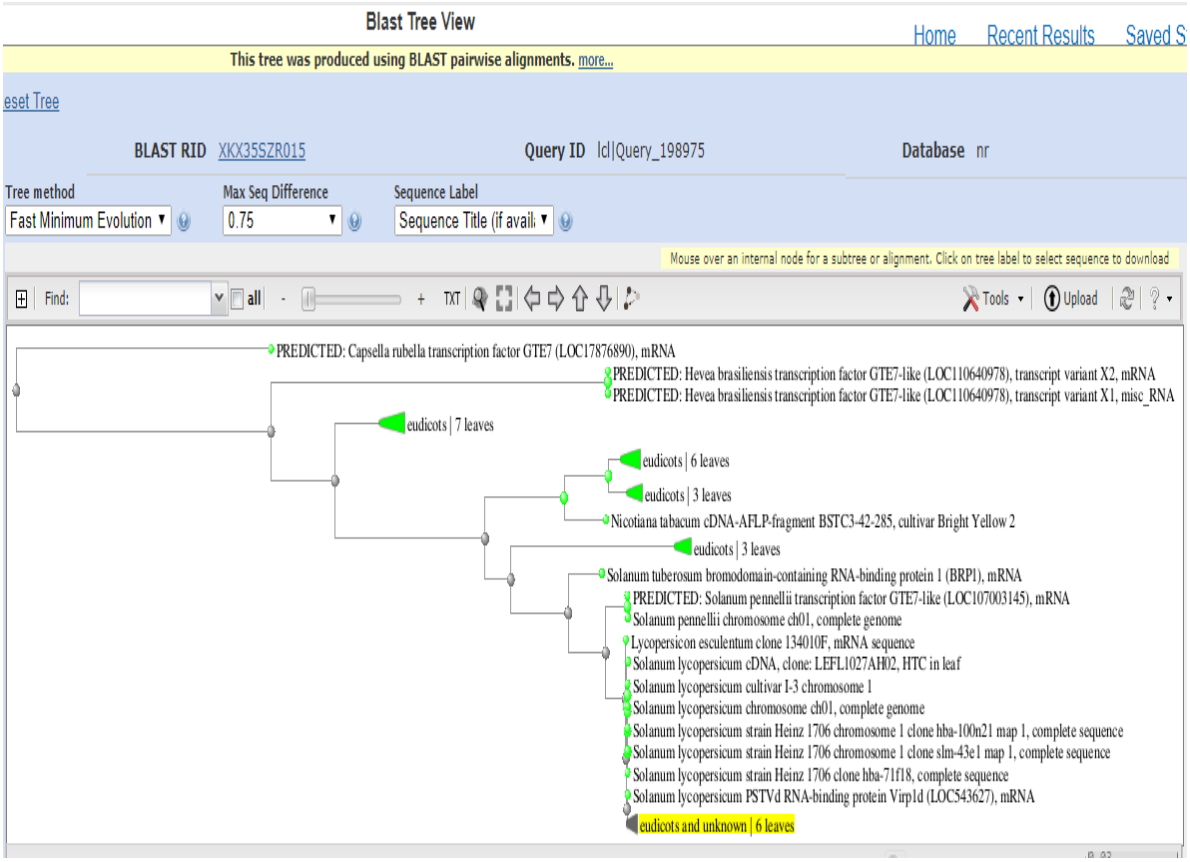

Fig 3. NCBI blast tree results of PSTVd FASTA showing 100% and 99% similarity with other reported sequence.

Sequencing

The presence of PSTVd was confirmed by BLAST and sequencing. The obtained FASTA sequences show 100% similarity with other reported accessions # such as AJ249595.1 in NCBI BLAST results. The amplified sequences were inserted into a vector according to protocol of Vachev *et al*. (2010) and sent for sequencing to BioBasic Company.

Amplification of PSTVd:

The PSTVd cDNA was further amplified with the primers designed by Boonham *et al*. (2004) and sent for sequencing. The amplified sequence also confirmed the presence of this deadly pathogen in our infected samples which we have collected from different places.

RESULTS

The PSTVd RNA from infected samples were extracted and analyzed. It was noted that by optimization of Trizol extraction methods we could achieve fine quality of RNA. This optimized Trizol method was also proved efficient in obtaining best quality of RNA and time saving. We have also stored the RNA in 2µL of Trizol reagent for future use. This was also another modification to store RNA without converting into cDNA. The prior addition of 2-Mercaptoethanol (1-2 µL per ml of Trizol reagent) also gave best quality PSTVd RNA. The amplified sequences gave positive, compact and distinct band for PSTVd (B). The Clear and sharp bands were obtained with PSTVd cDNA. The FASTA sequence also show matched with already reported sequence of PSTVd isolated from Tomato (Fig. 3).

DISCUSSION

The Isolation of PSTVd RNA has been reported earlier by many scientists and researchers in different countries (Bostan *et al*., 2003; Owen, 2007; Candresse *et al*., 2010; Qiu *et al*., 2016) The best of our knowledge and reports the present PSTVd RNA extraction is first report of presence of PSTVd from tomatoes in Pakistan. The previous discussed methods of PSTVd RNA isolation were also found superlative but those all need highly equipped Scientific Laboratories and environment, which is not possible in University Laboratories of progressing and low income countries in Pakistan. In contrast to viruses, these viroids are lacking of a proper protein-coat and no protein synthesis. This was the reason the serological detection approaches which were commonly used for viruses are not applicable to viroids (Diener, 1971). This presented work is reported is the modification of different researchers (Kasai *et al* 2013: Mackie *et al*., 2015). The present research is simple, easy and economic time saving isolation of PSTVd RNA. The modification of different extractions buffer (addition of b- Mercaptothion) and storing leaves in liquid nitrogen also gave best. It is worthy to note that isolated PSTVd RNA when amplified have shown complete match with NCBI BLAST results (Zhu *et al.*, 2002; Guner *et al*., 2012). These results have confirmed the presence of PSTVd in infected plants of tomatoes. Although PSTVd has been not reported in tomatoes Pakistan yet, there are a strong possible confirmation of PSTVd in infected plants of potato and tomato and causing PSTVd like diseases. This research could serve as a basic solid reference for planning and designing more explanatory research in viroids functions and structures in Pakistan. We have made successful attempts to attempts to isolate viroidal RNA from their infected host plants resulted in new optimized Trizol methods, which could be increasingly used for detection of viroids from plants. The attack of pathogens effects the economy of agricultural countries like Pakistan. It is need of time to diagnose and identify actual cause of plant diseases (Ding and Itaya, 2007) The infection symptoms of virus and viroids often confused with each other (Di Serio, 2007). In present work the symptoms of PSTVd are clearly elucidated and distinguished. The previous reports of PSTVd RNA instability were also taken into accounts (Kolonko *et al*., 2006; Ling *et al*., 2009) and storing condition of PSTVd RNA and cDNA were also improved during present research. The shelf and storage of RNAs life was also improved during our work. The knowledge and findings about PSTVd has been discussed a lot during last decades in European countries, However more need to be done, Like how this pathogens overtakes host plants to take advantage of them. The present work would open up new avenues and eras of pathogen and disease management in Pakistan.

Conclusion

The study has proved to be an efficient method for extraction of PSTVd RNA, its cDNA synthesis, amplification and storage from infected plants like tomato. The research would also provide a streamline study for future identification, control and management of PSTVd.

Conflict of Interests Statement

Authors declare that there is no conflict of interest for publishing this study.

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