RT-PCR detection of *Potato leaf roll virus* (PLRV) in aphids from Northern and North-Eastern India

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Abstract

A reverse transcription polymerase chain reaction (RT-PCR) assay was developed to detect Potato leaf roll virus *(PLRV) in aphids collected from potato fields. Total RNA was isolated from aphids and cDNA was synthesized with the help of random primers. In-house optimized PCR protocol for detection of PLRV targeting coat protein (CP) gene was adopted. Primers targeting cytochrome oxidase I (COI) were designed and PCR conditions were optimized so as to match with detection protocol of PLRV. However, before adopting the above optimized technique for routine detection of PLRV in aphids, the respective PCR amplified genes of PLRV (CP) and aphid (COI) were gel eluted and sequenced directly. The BLAST results revealed that both the queries showed a homology of 99 per cent each with PLRV and* Myzus persicae. *By adopting the developed RT-PCR technique, PLRV was successfully detected in the aphid samples collected from Shillong (Meghalaya) and Jalandhar (Punjab). Hence, this optimized technique was used to screen the aphids collected from different geographical locations for their viruliferousness with respect to PLRV. However, the virus was not present in aphids from other locations.*

Keywords: RT-PCR, PLRV, coat protein, mt COI, aphid, *Myzus persicae*

Introduction

Potatoes are infected by many viruses under field conditions during growing season. Among the important viruses infecting potato plant, *Potato leafroll virus* (PLRV) is one of the most significant viruses with world-wide distribution and causes yield loss up to 60-75%. (Paul Khurana, 1999). PLRV belongs to Polerovirus (family Luteoviridae) and the virions are isometric in shape with 26 nm in diameter. They generally occur in very low concentration and exclusively limiting to phloem cells and it contains positive sense single stranded RNA with 5.9 kb long. In some cultivars of potato, it severely affects the quality by causing internal damage to tubers (Nagaich *et al.,* 1974). Plants infected with PLRV experience chlorosis and upward rolling of young leaves, which is more apparent in most of the potato cultivars. The plants from infected tubers show stunting of the shoots with upward rolling of leaflets (basal leaves), which become rigid and leathery (Rodriguez and Jones, 1978).

Over 50 aphid species are known to be vectors of a large number of persistent and non-persistent viruses in potato (Ragsdale *et al.,* 2001). PLRV is transmitted primarily by green peach aphid (*Myzus persicae* Sulz.) in a persistent manner. Although, the acquisition and transmission of virus is a prolonged process, viruliferous aphids can remain infectious for their entire life (Bagnall, 1988); therefore PLRV can spread to longer distances by wind-borne winged aphids (Peters and Jones, 1981).

In order to understand the epidemiology of PLRV and subsequently to develop management strategies, more sensitive and reliable technique for identification of viruliferous aphids is very much essential. Moreover, detection of viruses in aphids is a challenging job because the size of the aphid is very small and the titre of the virus will also be very low. However, nucleic acid based detection technique, mainly reverse transcription polymerase chain reaction (RT-PCR) is an efficient and reliable technique for detection of potato viruses (Singh and Nie, 2003).

A false negative result may be produced in RT-PCR, even though the plants or aphids carry the infection (virus). In plants, generally housekeeping genes like 18S ribosomal RNA, nad2 mRNA (Du *et al.,* 2006), elongation factor 1-á (ef1á) gene in potato (Nicot *et al.*, 2005, Jeevalatha *et al.,* 2012), nad5 gene (mitochondrial mRNA) of apple (Menzel *et al.,* 2002) were used as internal controls to overcome the problem of false negative. With respect to aphids, poor quality of isolated RNA from aphids as they are small in

size or unintentional operational errors may lead to false negative results. Therefore, use or co-amplification of a universal internal control *i.e.*, cytochrome oxidase I (COI) of aphids with the viruses of interest would rule out the possibilities of false negative results and improves the reliability of the test (Simon *et al.*, 1994; He *et al.*, 2006). To the best of our knowledge, no literature was available on detection and identification of PLRV in aphids. Hence, a study was designed to optimize the detection protocol of PLRV in aphids along with COI as internal control by RT-PCR.

Materials and methods

Collection of aphids and total RNA isolation

Aphids were collected randomly from potato fields (Figure 1) from six different geographical locations, *viz*., Shimla (Himachal Pradesh), Jalandhar (Punjab), Shillong (Meghalaya), Arunachal Pradesh, Sikkim and Nagaland and samples were preserved in 70% ethanol stored at room temperature. The aphids were taken out from ethanol, air dried and transferred to clean eppendorf tubes. The total RNA was extracted using Axygen Mini Total RNA extraction kit (Axygen, USA) and stored at -20°C.

Designing of primers

Primers specific to coat protein gene (CP) of PLRV with an expected amplicon of ~492 bp along with optimized PCR protocols were adopted in this study. However, primers specific to mitochondrial cytochrome oxidase I (COI) region of aphid (internal control) were designed using Primer 3 software based on published sequences from NCBI data base and the same were synthesized and obtained from GenoBioscience Pvt. Ltd. The specificity of the designed primers was reconfirmed by BLAST analysis and the best combinations of primers were selected for further optimization of the technique (Table 1).

Reverse transcription (cDNA synthesis)

Total RNA isolated was used for c-DNA synthesis by using random primers with Revert Aid ™ c-DNA synthesis kit from Fermentas Life Sciences. The reverse transcription (RT) mixture *i.e.*, 4.0 µl of 5x buffer, 2.0 µl of 10 mM each dNTP mix, 1.0 µl of 20 U/µl RNase inhibitor, 1.0 µl of 0.2µg/µl Random primer, 6.0 µl of template RNA, 1.0 µl of 200U/µl RT enzyme and 5.0 µl of Rnase free water (to make up the volume), were added to provide a final volume of 20 µl. All the reactions were set up on ice cold condition to avoid premature cDNA synthesis and minimize the risk of RNA degradation. The reaction mixture was mixed and incubated at 25°C for 5min, 42°C for 59 min and 75°C for 10 min.

Optimisation of PCR protocol

The in-house optimized RT-PCR protocol was adopted for detection of PLRV in aphids and with respect to COI the PCR protocol was optimized in this study. PCR was carried out in a thin walled 0.2ml PCR tubes (GeneAmp PCR 9700 system from Applied Biosystems, USA) with reaction mixture of 20 µl containing 2.5 µl of 10x Taq buffer A, 1.5µl of 2 mM dNTP mix, 1 µl of 10 pM downstream and upstream primers each, 2 µl of cDNA (template), 1 µl of 1

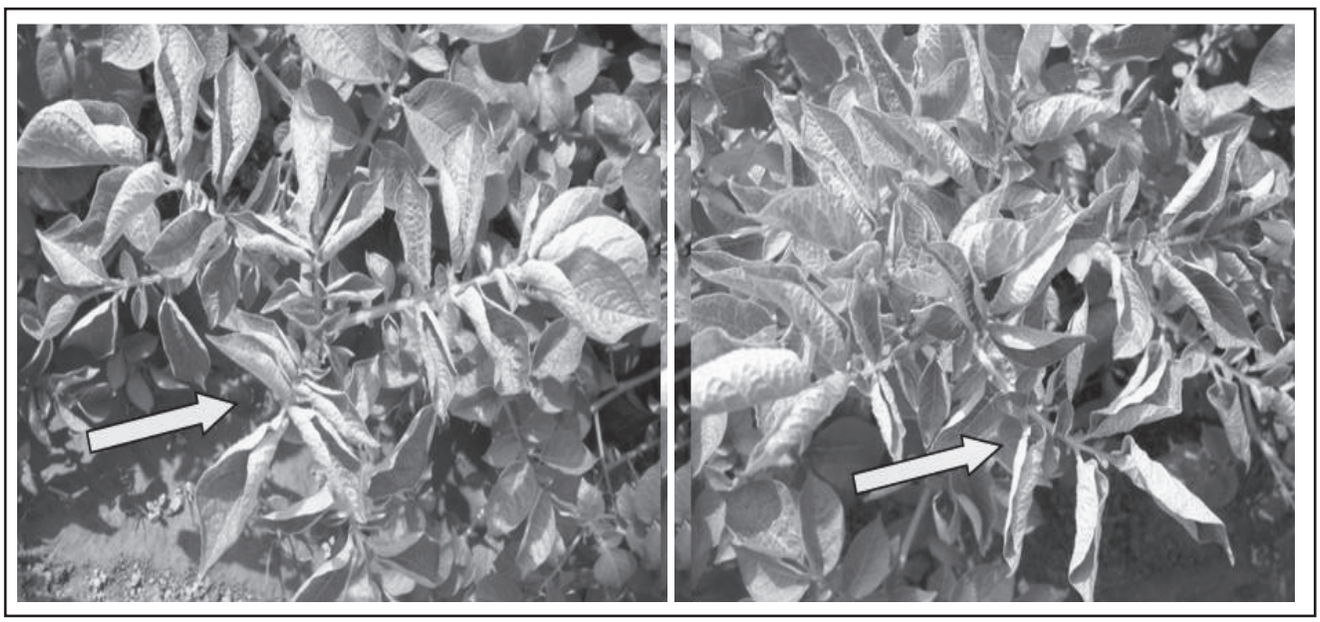

Figure 1. Potato plants showing upward rollng of leafs due to the infection by *Potato leafroll virus*

Table 1. Details of primers to detect PLRV and amplify COI region of aphids

Primer name	Primer sequence	Amplicon Size	Target
PLRV-FCP	CTAACAGAGTTCAGCCAGTGGTTA	~ 492bp	*Potato leaf roll virus*
PLRV-RCP	CGGTATCTGAAGATTTTCCATTTC		
ApCOI-1F	TCGGGTATAATTGGATCATCA	~ 579 bp	COI region of aphids
ApCOI-4R	TCCTCCTGCTGGGTCAAA		

U/µl of Taq DNA polymerase and 11 µl sterile nano pure water. Amplification was carried out to amplify COI region of aphids as follows: initial denaturation at 94^{0}C for 2 min, 35 cycles of denaturation at 94^{0}C for 30 sec, annealing at 56^{0}C for 45 sec, extension at 72^{0}C for 1 min; final extension at 72^{0}C for 5 min. The reaction mixture was loaded onto 1 per cent agarose gel and PCR amplicons were visualized on a UV-transilluminator and the primer pair showing expected size without any multiple bands was selected. The primer pairs were exposed to different annealing temperatures *i.e.*, 58, 60 and 62°C on a gradient PCR. The primers along with annealing temperature, which suited to PLRV was selected for further study. All these reactions were carried out as per the above master mix and PCR conditions with change in annealing temperature.

PCR amplification and sequencing of PLRV and COI

The above optimized PCR technique and selected primers were used to detect PLRV along with COI of aphids. The amplified products were gel eluted using gel elution kit and the eluted DNA was subjected to cycle sequencing reaction in a total volume 20 µl mix containing 3 µl of reaction mixture, 2 µl of 5x sequencing buffer (Big dye® Applied Biosystem, UK), 4 µl (0.8 pmol) of selected primers and 8 µl of eluted DNA. The cycle sequencing was performed with following conditions *i.e.*, 96°C/10 sec, 50°C/5sec, 60°C/4min for 25 cycles in GeneAmp PCR 9700 system (Applied Biosystems, USA). The cycle sequenced product was purified, denatured at 95 ^{0}C for 2 min and was chilled immediately on ice. The denatured product was sequenced with the help of 3500 Genetic Analyser, Applied Biosystems (Hitachi). The sequences obtained were aligned, assembled and confirmed by subjecting to analysis by using BLAST algorithm available at http://www.ncbi.nim.nih.gov. This technique was further used to screen the field collected aphids from six diverse geographical locations for the presence of PLRV.

Detection of PLRV in aphids

The collected aphids from different geographical locations were processed and RNA isolation, cDNA synthesis was carried out as mentioned earlier. cDNA was used template to detect the presence of PLRV in aphids by adopting the optimized PCR protocol.

Results and discussion

RT-PCR amplification

The total RNA isolated from aphids was used for synthesis of complementary DNA strand (c-DNA) wherein random primer was used as it greatly affects the subsequent PCR amplification. Therefore, commercially available random primer was used and was found more reliable in this PCR amplification studies. Primers specific to COI region of aphids were examined to check its feasibility by RT-PCR amplification. The results revealed that all the three primers were able to amplify COI region (~579 bp) with some non-specific bands or multiple bands (Figure 2) illustrating the necessary to optimize the primers with respect to annealing temperature.

Optimization of annealing temperatures

The primers specific to COI region of aphids were exposed to test the efficacy and specificity by exposing them to varying annealing temperatures of 58, 60 and 62°C. The results indicated that the primer pair ApCOI-1F & ApCOI-1R and ApCOI-1F & ApCOI-4R at 62°C successfully amplified a sharp amplicon of ~579 bp without any multiple bands. However, the primer ApCOI-1F and ApCOI-4R at 62 ^{0}C (Figure 3) was selected for further studies since it was showing a consistent and sharp amplicon of expected size without any non-specificity. It also matches with the RT-PCR detection protocol of PLRV.

PCR amplification and sequencing of PLRV and COI

The optimized PCR protocol was able to detect and amplify PLRV and COI with an expected amplicon of ~492 and ~579 bp, respectively. The results illustrate that the optimized RT-PCR along with selected primers can be used further to detect PLRV from a given population of aphids. However, before applying this technique to screen aphids for the presence of PLRV, the above RT-PCR amplified product was gel eluted and sequenced for confirming the reliability of the findings. The sequences obtained were aligned in BLAST analgorithm

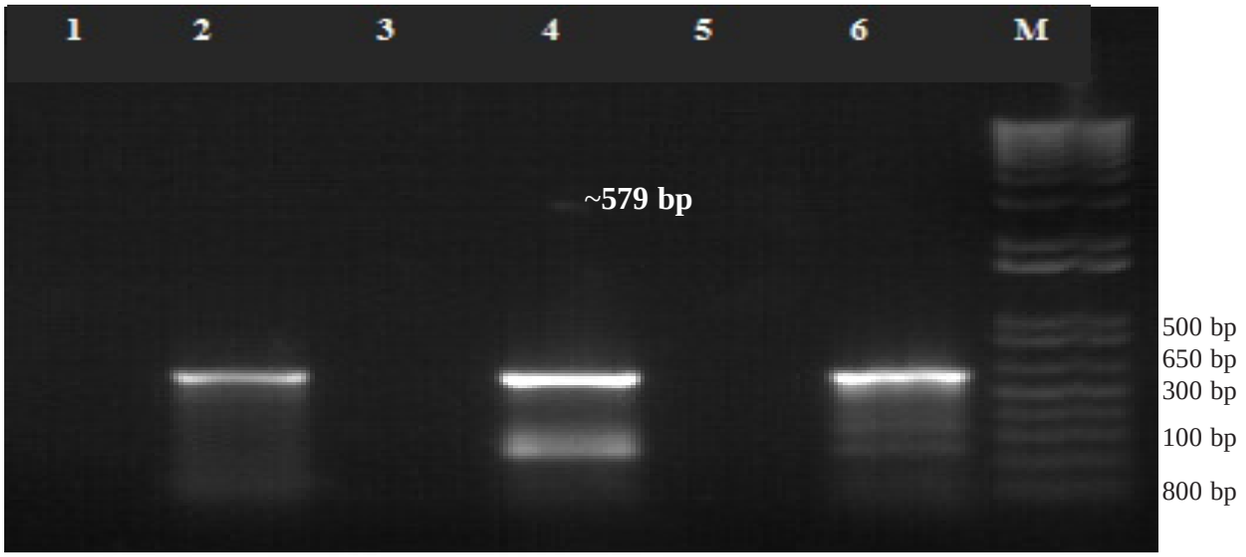

Figure 2. Selection of primer pair for amplification of COI in aphids. M-1 Kb plus ladder, Lane-1,3,5 are water controls and 2,4,6- are MP-1F1R , MP-1F2R & MP-1F4R at 56°C

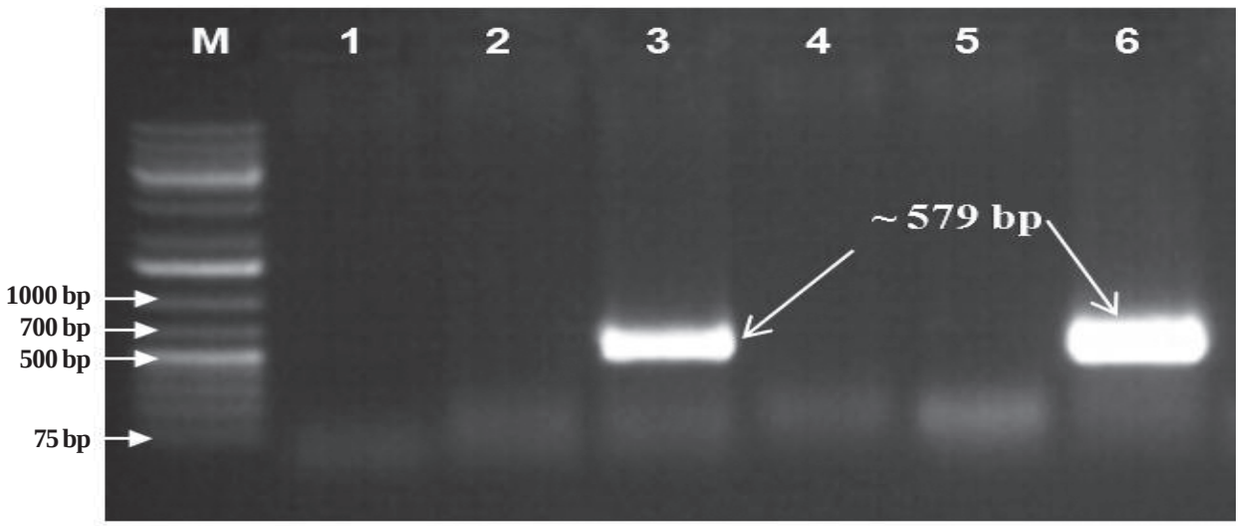

Figure 3. Optimization of annealing temperature. M-1 Kb plus ladder, Lane -1,2,3 1F!R and 4.5.6- are 1F4R at 58, 60 and 62°C, respectively

and the results revealed that the query (COI) was showing 99% homology with reported COI region, whereas, the coat protein gene of PLRV has a homology of 99% with reported PLRV from different geographical locations. Therefore, from the above finding, it is particularly evident that the optimized PCR technique can be further adopted as a robust technique to screen aphids for the presence of PLRV.

Detection of PLRV in aphids

The aphids collected from different geographical locations of India were processed to detect the presence of PLRV through RT-PCR and the results revealed that aphids collected from Shillong and Jalandhar (Figs. 4, 5 and 6) were positive for PLRV indicating that the aphids were carrying the virus while rest of the samples were negative.

In this study, RT-PCR reaction was optimized to detect PLRV in aphids. The results revealed a sharp amplicon of PLRV (~492) and COI (~579 bp) with optimized PCR conditions indicating the successful optimization of the technique to detect PLRV in aphids. The results were confirmed by repeating the reaction for consistency. These

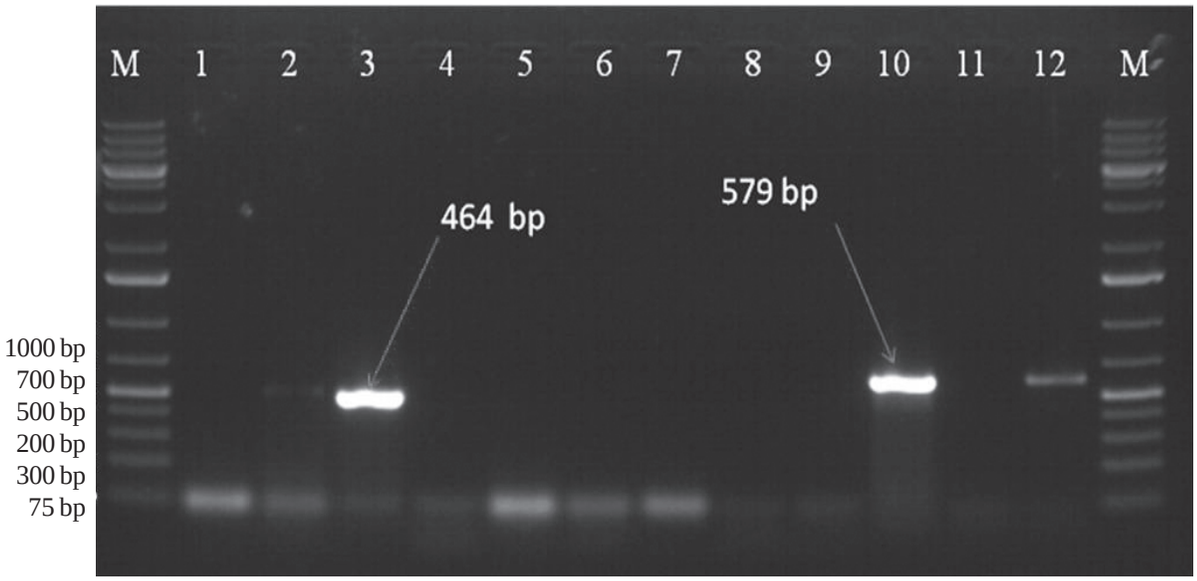

Figure 4. Detection of PLRV in aphids. M-1 kb plus, Lane1-water control (PLRV), Lane 2-healthy control (PLRV), Lane3-Positive control (PLRV) from Shillong, simillarly Lane 4 to 6 from Shimla, Lane 7 to 9 from Meghalaya. Lane10, 11 & 12 are IC for the samples from Shillong , Shimla and Meghalaya, respectively

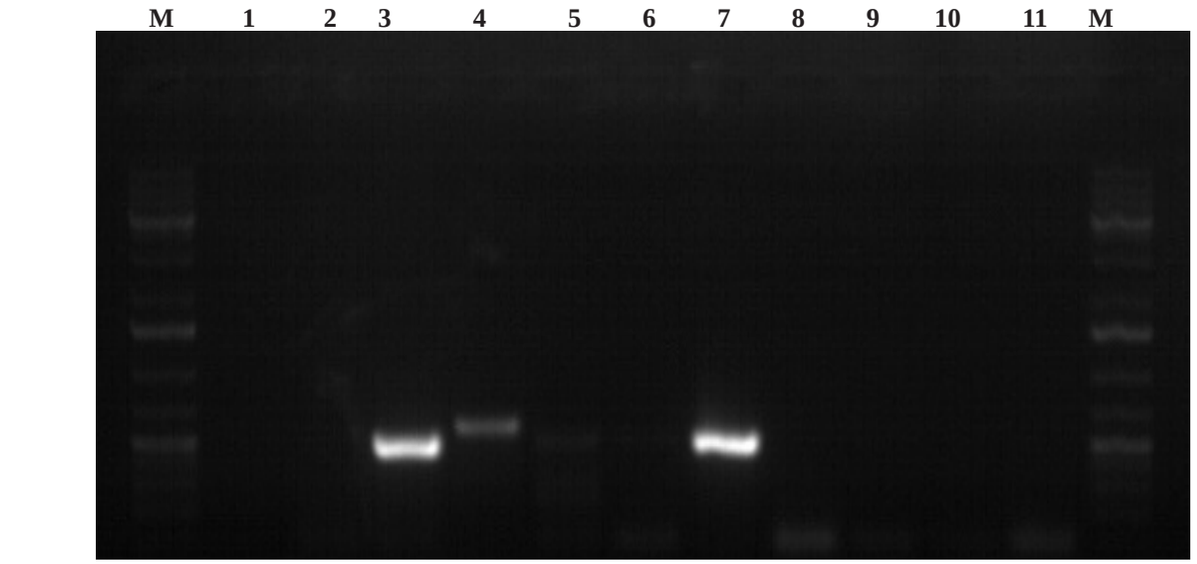

Figure 5. M-1 kb plus ladder, Lane1-water control, 2-healthy control, 3-Positive control, 4-IC, 5- aphids from Shimla, 6- 7 Jalandhar, Lane 8, 9, 10 & 11 are aphid samples from Meghalaya, Sikkim, Arunachal Pradesh and Nagaland, respectively

findings are in accordance with Singh *et al.* (1995) who developed RT-PCR based protocol to detect PLRV from aphids stored for one year in 70% alcohol; a triplex RT-PCR based protocol to detect PVY and PLRV from aphid (He *et al*., 2006) and very recently an immunocapture reverse transcription polymerase chain reaction (IC-RT-PCR) and RT-PCR to detect the virus in aphids (Ahouee *et al.*, 2010). However, in this study the commercially available random hexamer primer was used to synthesise cDNA and was found successful in further PCR reactions as the type of primer has a great influence. This eliminates the necessity of using virus specific antisense primers for each virus and also reduces the cost and time of RT-PCR procedure as a whole. These results are in accordance with the findings wherein it was reported that use of a common reverse primer, eliminates the need of a virus specific antisense primers for each viruses

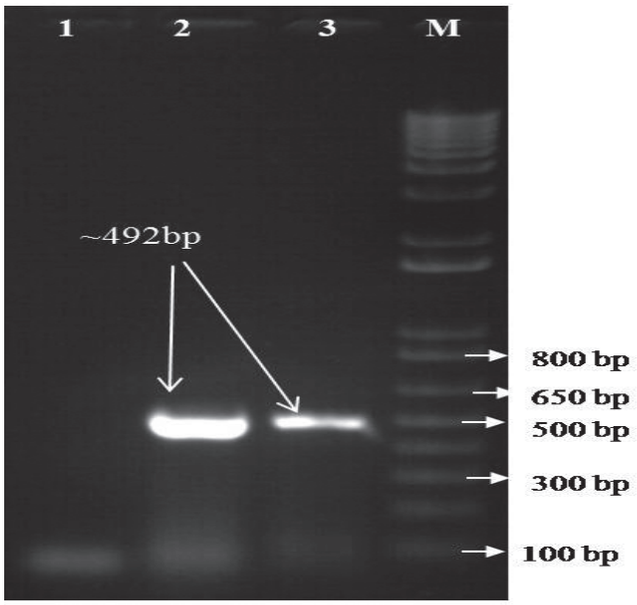

Figure 6. Lane 2 and 3-PLRV from Shillong M-100 bp ladder, Lane1-water control

(Singh *et al.*, 2000) and it was observed that the detection sensitivity is less when cDNA synthesis is primed with specific rather than random primers (Nie and Singh, 2001).

Many a times, in nucleic-acid based detection, specially reverse transcription polymerase chain reaction leads to false negative results due to which it becomes almost impossible to conclude with certainty whether the given aphids are carrying the virus or not. Similar to plant internal controls (Jeevalatha *et al.*, 2012; Raigond *et al.*, 2013) it is very much essential to have internal controls targeting the genome of aphids (COI mRNA) to avoid the false negative results in RT-PCR assays. Ampliûcation of internal control indicates the reliability of the results as it was proved by He *et al.* (2006) that co-amplification of aphid mRNA from the cytochrome *c* oxidase subunit I serve as an indicator of RNA quality. Despite, the variability exists in COI, it is believed to be conserved among various species of aphids (Simon *et al.*, 1994). Therefore, the primers specific to COI region of aphids were designed and selected a primer pair *i.e.*, ApCOI-1F & ApCOI-4R by optimizing the PCR conditions especially with respect to annealing temperature (Majumder *et al.*, 2008; Santosh *et al.*, 2012, 2013; Raigond *et al.*, 2013). The selected primer pair with optimized PCR conditions were able to amplify a specific and desired amplicon of ~579 bp of aphid mRNA. Hence this primer pair was used in RT-PCR detection of PLRV along with internal control.

The technique was reconfirmed by sequencing both the amplified fragments and confirming the specificity with BLAST analysis. It also gave an indirect evidence that aphids collected and used in this study was identified as *M. persicae*. Hence, it is clearly evident from these findings that optimized PCR technique can be further adopted as a robust technique to screen the aphids for the presence of PLRV. So the optimized protocol was used to detect PLRV wherein, the virus was detected from the aphids collected from Shillong (Meghalaya) and Jalandhar (Punjab) indicating viruliferous nature of aphids and presence of the virus in the potato fields whereas aphids from other locations were not carrying PLRV. This type of tests can indicate the health status of breeder and nucleus potato seed crop in field for better decision making.

The above study illustrates the feasibility of adopting the developed RT-PCR protocol to detect PLRV in aphids collected from infected plants/ sticky traps/ suction traps and stored in 70% ethanol. Even though, it is evident from the literature that the technique for PLRV detection in aphids is available but, the combination of including internal control, sequencing, sequence analysis and validating the protocol is not available, which intern elevates the confidence with high reliability. Hence we report here that the RT-PCR based detection of PLRV along with internal control has potential value for epidemiological studies and might also help in elucidating virus-vector interactions.

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