African Journal of Biotechnology Vol. 12(11), pp. 1196-1202, 13 March, 2013
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB12.1951
ISSN 1684–5315 ©2013 Academic Journals

Full Length Research Paper

Molecular characterization, cloning and sequencing of coat protein gene of a Pakistani potato leaf roll virus isolate and its phylogenetic analysis

Md. Belal Hossain*, Idrees Ahmad Nasir, Bushra Tabassum and Tayyab Husnain

National Centre of Excellence in Molecular Biology (CEMB), University of the Punjab, 87-West Canal Bank Road, Thokar Niaz Baig Lahore, 53700, Pakistan.

Accepted 23 January, 2013

Total RNA was extracted from potato leaf roll virus (PLRV) positive potato plants and complementary DNA were synthesized. Reverse transcriptase polymerase chain reaction (RT-PCR) based detection conditions were optimized by using coat protein (CP) gene specific primers. A 346 bp amplicon of PLRV- coat protein (CP) gene was amplified. Amplified CP-gene of PLRV was cloned in TA cloning vector, P^{CR} ® 2.1. The clone was confirmed subsequently through restriction digestion analysis. PCR amplification used cDNA clone as a template and nucleotide sequencing. Expected nucleotide sequence of amplified PLRV-CP gene show homology of 94 to 97% when compared to the sequences already reported in GenBank database. This explored novel PLRV-CP gene was submitted at NCBI GenBank for characterization of PLRV Pakistani isolate (Accession No. JN039286). Phylogenetic analysis was also carried out and tree was made by using MEGA 4.0.

Key words: Potato, potato leaf roll virus (PLRV), coat protein (CP) gene, reverse transcriptase polymerase chain reaction (RT-PCR), PLRV-CP Pakistani isolate.

INTRODUCTION

The genus *Solanum*, to which cultivated potato belongs, is large consisting of about 1000 species. More than 200 species of potato have been found, but just eight are cultivated and have been cultivated for the last 2000 years (Smith, 1977). Among the most important food crops in the world, Potato (*Solanum tuberosum* L.) is in the fourth position after wheat, rice and maize. In the world, potato is cultivated over 53 666 thousand ha of lands and production is 752 632 thousand tonnes and in Pakistan, 175 thousand ha of lands was cultivated and production was 3 420 thousand tonnes in 2009 (FAOSTAT, 2010). It is consumed by over one billion people worldwide, and is the part of diet of a billion consumers in the developing countries. In developing countries, viral diseases are one of the major causes of low yield of potatoes and their control requires the development of appropriate, sensitive and reliable detection methods along with the study about their persistence in various plant parts (Salazar, 1994).

Potato leaf roll virus *(PLRV)* causes devastating effects on potato production. This virus initially affects the aerial tissues of potato plants causing the stem and apical leaves to roll. PLRV-infected plant leaves become similar to the rattling of parchment paper. The diseased plants produce fewer and smaller tubers than the normal plants resulting in significant yield reduction and some varieties may show "net necrosis," small brown strands of discolored tissue extending throughout the stem end of the tuber after a month in storage. PLRV differs from other potato viruses, because it is exclusively found in phloem tissues and causes necrosis and abnormal callose accumulation in the vascular system. PLRV is a

*Corresponding author. E-mail: mbhossainsau@yahoo.com. Tel: +92-03238416125 or +88-01711988444. Fax: +88-02-8155800.

persistent virus and vectored by several aphid species; the green peach aphid (*Myzus persicae*) is the most important one of them (Spooner, et al., 2005).

PLRV is distributed worldwide in potato growing areas. Quality of seed tubers is badly affected due to the development of phloem necrosis. It is a popular potato virus in Pakistan and widely distributed in the country, with high incidence of 15 to 65% (Mughal et al., 1988). It is proved by a survey study on PLRV, and in that study, they were highly suggested to implement planting cultivars that are virus resistant for the control of PLRV in potato crops (Jan and Khan, 1995). PLRV is a member of the genus *Polerovirus* and belongs to the family Luteoviridae. It has a monopartite, non-polyadenylated RNA genome of ~6 KB. This virus possesses a 5.9 kDa genome consisting of a single strand RNA molecule with plus orientation that encodes six open reading frames (ORFs) and three of these frames are located near the 3 end and encode, through sub genomic RNA molecules, the 23 kDa coat protein (*CP*) (Van Der Wilk et al., 1989, 1997), a 17 kDa fluorimetric movement protein (MP) (Sokolova et al., 1997) and a 56 kDa protein involved in the virus/vector aphid interaction (Chay Ca et.al., 1996).

A Scottish PLRV isolate has been published more than a decade ago as a complete genomic RNA sequence (Mayo et al., 1989). The genome size of that particular isolate was 5 987 nucleotides and contained six large open reading frames. The ORF 3' encodes a *CP* about 23 kDa in size. Since then, CP genes and their complementary DNAs have been published for several PLRV isolates throughout the world. Though, a very high level of sequence homology has been observed among geographically distinct stains of PLRV, key changes in sequences of both nucleotide and amino acids have been reported. No published information is so far available regarding the PLRV-CP gene sequences of Pakistani isolate. The goal of this study was to detect and characterize PLRV through RT-PCR amplification, cloning and sequencing of *CP* gene for characterization of PLRV Pakistani isolate. Through this study, a reliable RT-PCR based molecular detection method was developed for local strain of PLRV and a Pakistani isolate of PLRV was found on the basis of nucleotide sequencing from cloned cp gene. The sequence identity analysis of the cloned PLRV -*CP* gene was used to assess homologies among several PLRV isolates reported in Gene bank database.

MATERIALS AND METHODS

PLRV -infected plants were collected from field, based on typical symptoms of PLRV pale in color and rolled leaves. Plants were maintained through single node culturing on MS basal media. Cultured plants were placed at 20±2°C under 16 h photoperiod.

Primer designing

Primers were designed using primer 3 software (version 0.4.0) (Steve and Helen, 2000) for amplification of PLRV-*CP* gene. For this purpose, PLRV - CP gene sequence was retrieved from GenBank (Accession number NC b001747 verified on 2010/04/15). Multiple alignments were done by using Clustal-W (version-2.0), (Larkin et al., 2007) and conserved regions were identified. Primers were designed to target the conserved region and its sequence was tested in silicon using BLAST on GenBank to minimize the likelihood of non-specific amplification. Primers having 3′-end sequence showed no sequence homology with other viruses, or other sequences in GenBank were selected. Primer oligos were synthesized from CAMB primer synthesis lab, Lahore, Pakistan.

RNA extraction

Total RNA was extracted from PLRV-infected potato leaves by using TRIzol reagent (Invitrogen) according to manufacturer's instructions. RNA was precipitated with isopropanol and washed with 70% ethanol. Finally, RNA was resuspended in 20 µL of DEPC H_2O.

Complementary DNA (cDNA) synthesis

cDNA was synthesized using "First Strand cDNA Synthesis kit" (Fermentas). Complementary DNA was prepared by mixing 4 µL of total RNA, 1.0 µL of PLRV-346 reverse primer (10 pmoles) and nuclease-free water to a final volume of 11 µL, mixed gently and spun down for 3 to 5 s in a microcentrifuge. After incubation at 70°C for 5 min and chilling on ice, 4 µL 5x reaction buffer, 1 µL Ribolock™ (Ribonuclease-inhibitor) (20 ug/µL) and 2 µL of 10 mM dNTPs was added and mixed. Incubation was done at 37°C for 5 min and 2 µL M-MuLV Reverse transcriptase (20 ug/µL) was added to make a total of 20 µL reaction volumes. Finally, incubation was done at 37°C for 60 min and the reaction was stopped by heating at 70°C for 10 min and chilling on ice immediately.

Reverse transcriptase polymerase chain reaction (RT-PCR)

cDNA was used as template and 346 bp fragment was amplified from 3'coding sequence of PLRV using primer pair Forward 5'-CAGGCGCCGAAGACGCAGAA-3' and Reverse 5'-TTTGGCGCCGCCCTTCGTAA-3'. The total volume of 20 µL PCR reaction contained 10 × PCR buffer ($MgCl_2$ 2.5 mM, 10 mM Tris-HCl pH 8.0), 1 mM of each deoxyribonucleoside triphosphate (dNTP), 10 pmoles of each forward and reverse primer, and 1 unit *Taq* DNA polymerase. PCR amplification was performed by 35 cycles after initial denaturation at 94°C for 4 min in a thermal cycler starting with denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min and extension at 72°C for 1 min followed by final extension at 72°C for 10 min. The RT-PCR products were resolved on 1.5% agarose gel in 1X TAE buffer. 50 bp DNA ladder (Fermentas) was used as marker to determine the size of RT-PCR products. Gel was stained with ethidium bromide and visualized by UV illumination using Gel Documentation System (Gel Doc 2000, Bio-Rad, USA).

Amplified coat protein gene cloned in TA vector

RT-PCR products were resolved on 1% agarose gel and 346 bp fragments were cut for elution. Elution was done by using silica bead DNA Gel Extraction kit (Fermentas). The purified PCR product was ligated in TA cloning vector PCR®2.1 (Invitrogen) in 1:1 ratio. *Escherichia coli* strain DH5α cells were made before ligation reaction and stored at 70°C. Ligation mixtures were transformed in chemically competent *E. coli* strain DH5α cells by heat shock

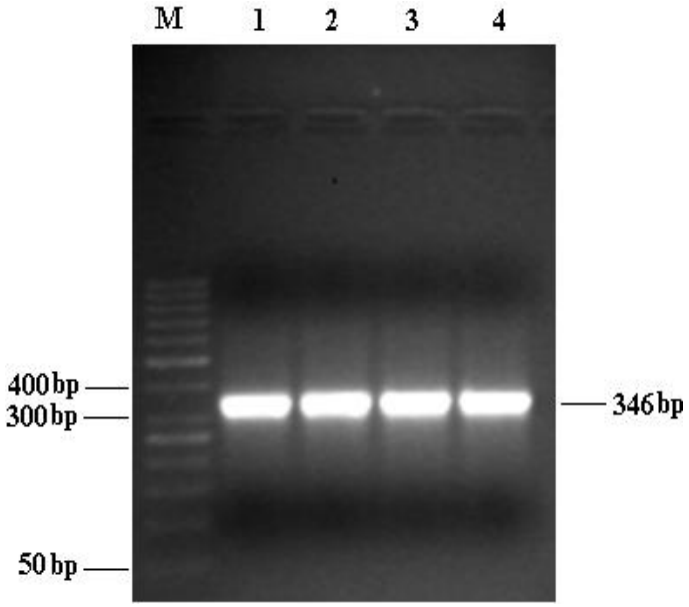

Figure 1. Amplification of PLRV-CP gene through RT-PCR. Fragment size of 346 bp was amplified using gene specific primers. M= 50 bp DNA ladder; lane 1-4 depicts PLRV -*CP* gene amplification at 346 bp.

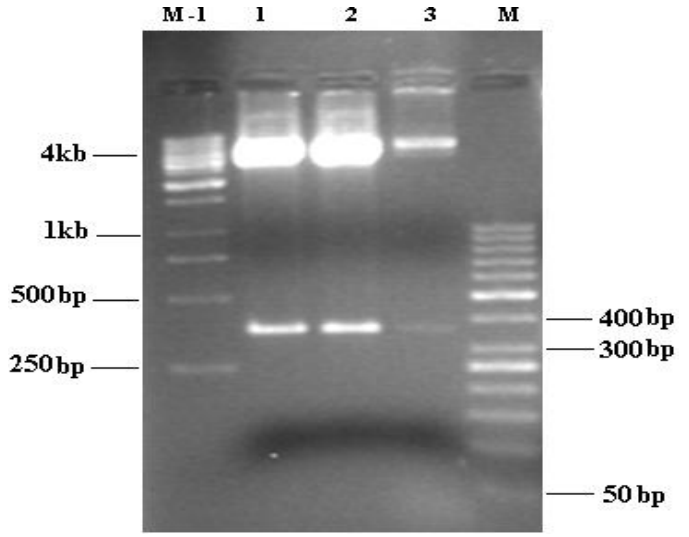

Figure 2. Restriction digestion with *EcoR* I enzyme. *EcoR* I digested two bands; one was the vector at~3.9kb while insert size was~346 bp. M-1= 1kb DNA ladder; lane 1-3 digested with *EcoR* I at 346 bp and M= 50 bp DNA ladder.

method according to manual protocol. Blue/white selection was used as a screening tool. Plasmid DNA was isolated from selected white colonies using GeneJet Miniprep plasmid isolation kit (Fermentas). Plasmid DNA was digested with EcoR1 enzyme and fractionated on agarose gel. Clony PCR was also performed using recombinant plasmid DNA as a template for confirmation of the insert.

Sequencing of PLRV-CP gene

Automated DNA sequencing system (ABI 3700) from applied Biosystems was used along with ABI PRISM genetic analyzer (Applied Biosystems, USA) using BigDye^{TM} terminator cycle sequencing Kit according to manufacturer's instructions. The PLRV-CP gene was sequenced using M13 primers (forward and reverse), respectively. The data was provided as fluorimetric scans, from which the sequence was assembled using the Sequence Navigator software. The nucleotide sequence was analyzed using CHROMAS sequence analysis software version 2.0 and compared with PLRV sequences reported in GenBank database.

Sequence comparison and phylogenetic analysis

Homology studies were done through standard nucleotide-nucleotide BLAST (Basic Local Alignment Search Tool) software available at NCBI web site (www.ncbi.nlm.nih.gov/home/BLAST). The multiple sequence alignment was done using Cluster W multiple sequence alignment programs integrated in MEGA4.0. Phylogenetic analysis was done by MEGA 4.0 (Tamura et al., 2007). Maximum parsimony method was selected to construct phylogenetic tree. In “compute tree” option of the software, we selected “consensus tree” and then software returned the given phylogenetic reconstruction tree.

RESULTS

Molecular detection of the PLRV-CP gene via RT-PCR

PLRV -infected potato plants were selected on the basis of typical symptoms rolled stem and stand upright leaves for RNA extraction. Synthesized cDNA was used as a template in PCR amplification, using gene specific primers, designed to amplify 346 bp fragment of PLRV from the coat protein gene region. Annealing temperature was optimized at 55°C *via* gradient PCR. Total volumes of RT-PCR products were resolved on 1.5% agarose gel along with 50 bp molecular weight marker. The expected size of the PCR product was ~ 346 bp. Amplification was sharp and clear as depicted in Figure 1.

Cloning of PLRV-CP gene into TA-clone vector P$^{CR®}$2.1 and isolation of recombinant plasmids

Amplified PLRV coat protein gene was ligated with 50 ng of P$^{CR®}$2.1 vectors in 1:1 ligation ratio. Chemically competent *E. coli* (DH5α strain) cells were used for transformation. Transformants were selected on LB agar plates containing kanamycin (50 ug/ml), X-Gal (40 ug/ml) and IPTG (40 ug/ml) for blue white colony selection. White colonies were analyzed by picking 10 to 15 single colonies and transformation into 5 ml LB-kanamycin (50 ug/ml) culture. The recombinant plasmid DNA was isolated by alkaline lysis method. Confirmation was done through restriction digestion with *EcoR* I restriction enzyme as clear from Figure 2. PCR amplification was also done using plasmid DNA as a template with PLRV346-FP and PLRV346-RP primers to amplify the 346 bp fragments of PLRV-CP gene which is clearly shown in Figure 3.

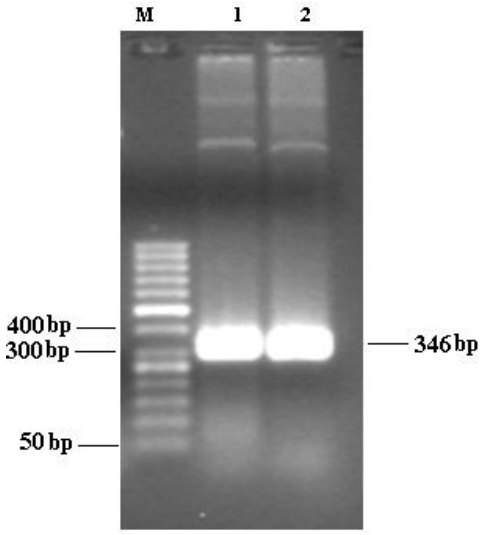

Figure 3. Clone PCR amplification, and plasmid DNA used as template with PLRV-CP gene primers. Two bands are depicted; upper one is the vector and lower one is the insert size was~346. M= 50bp DNA ladder and Lane 1 and 2 are clone PCR amplified at 346 bp.

PLRV-CP gene sequence analysis

Confirmed PLRV-CP gene clone was used as a template with oligonucleotide (M-13) primers for the full-length sequencing of the gene through automated DNA sequencing system. Sequence was compared with NCBI GenBank reported sequences. We BLAST our sequence with the existing database entries. In total, 99 sequences of coat protein genes from different PLRV isolate around the world were selected on the bases of homology. Minimum homology was 94% among the aligned sequences while the maximum was 97%. We submitted this sequence at NCBI GenBank data base as a PLRV coat protein gene sequence (CEMB-1 Lahore isolate, Pakistan) and GenBank provided the accession number [GenBank Accession No. JN039286]. Sequence of the isolate is shown in Figure 4.

Sequence comparison and assessing the genetic diversity through phylogenetic analysis

Multiple sequence alignment with the nucleotide sequence of our isolate (CEMB-1, Lahore, Pakistan) show considerable similarity with the other members of Luteoviridae family. That similarity can be viewed in the phylogenetic tree developed from multiple sequence alignment. Our isolate exhibited its maximum homology of 97% with Scottish isolate (Accession number EU717546.1), Mashad (Iran) isolate (Accession number FJ481108.1), and Canadian isolate (accession number D13954.1). With Indian isolate homology were 96% (Accession number GU256062.1) and 95% with Australian isolate (Accession number D13953.1). The minimum homology 94% was shown by only one isolate (Egyptian, Accession number GQ376029.1). It is clearly shown in the phylogenetic tree (Figure 5). There was not too much diversity among the globally found PLRV isolates. High similarity with other isolates depicts that CP gene sequence is also highly conserved among PLRV and can be used efficiently in molecular detection of this virus.

DISCUSSION

Potato is the most important food crop in the world and also in Pakistan. But the production is still very low in Pakistan as compared to other potato cultivation countries in the world. Potatoes are infected by several numbers of pathogen. The most important pathogens are the viruses; the PLRV is the most important one and a major treats in potato production (Gul et al., 2011). Presently, farmers use different insecticide for controlling this virus. Although, it cannot be directly control by chemical application and cause economic loss for farmers, producers and consumers. The use of resistance cultivars that would be resistant to virus infection is the only sustainable and environmentally friendly method of control of virus in potato crops. So far, there is no report to reproduce the biophysical and biochemical properties of PLRV in Pakistan. The introduction of the PCR has gained a wide popularity in many molecular applications including cloning, sequencing and site specific mutagenesis. The success of PCR depends upon a number of factors like template DNA/cDNA (RT-PCR), primers, dNTPs and magnesium ion concentration in the reaction mixture as well as choice of polymerase enzyme and primer's annealing temperature (Saiki et al., 1985).

In this study, the first objective was to develop a RT-PCR based detection protocol of PLRV for commercial purposes. Because RT-PCR provides great sensitivity than molecular hybridization like ELISA, it is a good alternative to other diagnostic methods with high speed diagnosis and requires reduce sample size. The availability of nucleotide sequences of many plant pathogens has made possible the development of PCR assays for the detection and diagnosis of several viriods, viruses and other pathogens. The RT-PCR has also been successfully utilized for the detection of plant viruses for example, grapevine virus A (GVA) from infected grapevine, apple scar skin and pome fruit virus and potato virus A (PVA) in dormant tubers (Haididi et al., 1990; Minafra et al., 1992; Haididi et al., 1993; Singh and Singh, 1998). Multiplex-RT-PCR (m-RT-PCR) has also been reported for the simultaneous detection of five potato viruses that is, potato virus S (PVS), potato virus X

1 CAGGCGCCGAAGACGCAGAATAGGAGGCAATTCGCCGCTCAAGAAGAACT GGAGTTCCCC
61 GAGGACGAGG CTCAAGCGAG ACATTCGTGT TTACAAAGGA CAGCCTCATT GGGCACTCCC
121 AAGGAAGTTT CACCTTCGGG CCGAGTCTAT CAGACTGTCC GGCATTCAAG GATGGAATAC
181 TCAAGGCCTA CCATGAGTAT AAGATCACAA GCATCTTACT TCAGTTCGTC AGCGAGGCCT
241 CTTCCACCTC CTCCGGTTCA TCGCTTATGA GTTGGACCCC CATTGCAAGT ATCATCCCTC
301 CAGTCCTACG TCAACAAGTT TCCAAATTAC GAAGGGGCGG CGCCAA

Figure 4. Nucleotide sequence of the PLRV *-CP* gene (CEMB-1 Lahore, Pakistani isolate, GenBank Accession No. JN039286). The sequence length was 346 bp.

Figure 5. Phylogenetic tree generated by MEGA 4.0. All the globally found PLRV isolates are much closed to each other as clear in the tree.

(PVX), PLRV, potato virus A and Y (PVA and PVY) and potato spindle tuber viroid (PSTVd) (Nie and Singh, 2000). Although in some studies, Real time PCR has been described for efficient detection of PLRV in dormant tubers (Agindotan et al., 2007; Mortimer Jones et al., 2009), but due to high costs of reagents and equipment involved in real time PCR, the method described in our present study is more applicable and cost-effective. The RT-PCR-based assay can be considered as a potential test for routine diagnosis because of its higher sensitivity and less time-consumption (Schoen et al., 1996). So, we concluded that this molecular technique would be a useful tool for epidemiological studies of PLRV and can be used for certification program as well as detection of foci of PLRV in potato crops as early as possible.

The other objective of this present study was cloning

and sequencing of PLRV-CP gene. We cloned PLRV-CP gene in molecular vector and cloned confirmation was done by restriction digestion, PCR amplification and its nucleotide sequencing. Sequence was compared with other PLRV isolates sequences reported in GenBank databases and showed considerable homology. We found the cloned PLRV-CP gene which was strongly related (94 to 97%) with all reported isolates of PLRV. It means sequence gene is relatively conserved with PLRV genome that is similar to previous reports (Kesse et al., 1990). High homology was observed with some European isolates as well as with Canadian isolates and somewhat distant relationship was observed with Australian isolate. In our study, 346 bp of the total coat protein gene size of PLRV was sequenced (GenBank Accession No. JN039286) and homology of the sequenced gene was studied with the already reported sequences in the gene bank database through Basic Local Alignment Search Tool (BLAST) software and it showed least homology, that is, 94% with only one reported gene sequence (Egyptian isolate gene bank accession number GQ376029.1) and maximum homology 97% with some European isolate (accession number EU717546.1), Mashad (Iran) isolate (accession number FJ481108.1) and Canadian isolate (Accession number D13954.1). With Indian isolate (accession number GU256062.1) and Australian isolate (accession number D13953.1), it showed 96 and 95% homology, respectively.

Similar results were found when compared with two Korean isolates of PLRV-RB and PLRV-CA. It showed closer relationship with Canadian isolate, 98 to 99% homology of several European isolates and somewhat lower degree of similarity (96 to 97%) between these isolates and Australian one have been reported (Martin et al., 1990; Joung et al.,1997). In another study, same results were gotten when Italian isolate was compared with the five European isolates along with the Australian one (Faccioli et al., 1995). Another two recent reports also showed similarity with our findings when compared with the nucleotide sequences and the deduced amino acid sequences of Indian isolates with the other PLRV isolates; they found 97 to 99% similarity at both the nucleotide and amino acid sequence level of other isolates (Mukharjee et al., 2003) and in case of Egyptian isolates, 95% homologous was found to those of other *Luteoviruses* at the nucleotide level (El-Attar et al., 2010).

Conclusion

On the basis of our findings, it is concluded that: 1) the novel gene and hyper variable region sequence of PLRV coat protein gene be explored, which may play a vital role in pathogen derived resistance, and 2) the portion of gene sequenced in the present study showed homology within the range of 94 to 97% with others PLRV-CP gene sequences reported in GenBank database and did not show 100% homology with any of the reported isolate. So, it is proved that this sequence may not have been reported before.

ACKNOWLEDGEMENTS

This work was supported by the Academy of Sciences for the Developing world (TWAS), Trieste, Italy and the National Centre of Excellent in Molecular Biology (CEMB), Lahore. Pakistan. The principal author also acknowledges the Sher-e-Bangla Agricultural University, Dhaka, Bangladesh, for granting study leave for PhD program.

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