Complete Genome Sequence of *Potato leafroll virus* Isolates Infecting Potato in the Different Geographical Areas of India Shows Low Level Genetic Diversity

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Abstract Five *Potato leafroll virus* (PLRV) isolates were collected from five states representing different potato growing parts of India. The ssRNA genome sequences of these isolates were determined. The genome comprised of 5,883 nucleotides and deduced genome organization resembled other PLRV isolates. About 97.6–98.7 % similarities was observed within the Indian isolates and were more close to European, Canadian, African, American and Czech isolates (95.8–98.6 %) than to an Australian isolate (92.9–93.4 %). These isolates were 43.7–53.1 % similar to other poleroviruses and 29.1–29.3 % to *Barley yellow dwarf virus*, a luteovirus. Out of five isolates, the isolate PBI-6 was recombinant one as detected by RDP3 software. Multiple sequence alignment of nucleotide and amino acid sequences of different ORFs indicated that the ORF 3 and ORF 4, corresponding to coat protein and movement proteins are more conserved than other ORFs. Amino acid changes specific to Indian isolates were observed and it was more in ORF 2 than in ORF 0, ORF 3 and ORF 4. This is the first report of complete genome sequence of PLRV isolates from India, which reveals low level genetic diversity.

Keywords *Potato leafroll virus* · Genome sequence · Indian isolates · Phylogenetic analysis · *Polerovirus*

Introduction

Potato leafroll virus (PLRV) is one of the most prevalent viral diseases of potato in India [14]. It is the type species of the genus *Polerovirus*, in the family *Luteoviridae* [13], a group of phloem limited plant viruses. The virus is tuber borne, not sap transmitted, transmitted efficiently by aphid in a circulative non-propagative manner [6, 16]. Yield loss normally ranges from 50 to 60 % in India [14, 16]. PLRV has small, isometric virions measuring about 24–25 nm in diameter. The genome consists of positive sense single stranded RNA with the viral genome linked protein (VPg) at the 5' end [12] and an OH group at the 3' end. The genome is divided into two parts by a small non coding RNA and harbours nine open reading frames (ORFs) numbered from 0 to 8 coding for proteins, P0–P7 and Rap1, respectively. Three 5'-proximal ORFs, which are expressed from the genomic RNA, encode the proteins, P0, P1 and P2. Five other ORFs are expressed by translation from two sub genomic RNAs (sgRNAs). Two structural proteins (P3, P5) and P4 are encoded by sgRNA1 and the sgRNA2 encodes two 3'-proximal proteins (P6, P7). ORF 1 harbours a small ORF, ORF 8 which encodes Rap 1.

P0 is involved in symptom development and acts as a suppressor of RNA silencing [18] and has functional motifs like F-BOX motif to overcome PTGS [17]. P1 is a proteinase containing polyprotein responsible for the release of virus encoded protein (VPg) [20, 21, 24]. P2 is translated by a rarely occurring ribosomal frame shift from ORF1 and carries the conserved motifs typical of RNA-dependent RNA polymerases (RdRp). P3, P4 and P5 correspond to the major capsid protein (CP), the putative movement protein (MP) and the read through domain (RTD), which is translated by suppression of the ORF3 stop codon. The P5 of luteoviruses has been proposed to play a role in

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interaction between the virus particles and receptors in the aphid vectors [7] especially the 5-terminal conserved half may be the site of vector specificity [4]. The functions of P6 and P7 are not known. But P7 is reported to have nucleic acid binding properties [1]. Rep1 is involved in virus replication [9]. Almost all types of modulation mechanism (frame shift, initiation bypass, termination suppression, production of subgenomic (sg) RNA and proteolysis of primary translation products) are used during the expression of the different ORFs (reviewed by [22]).

So far only the coat protein gene sequence of a single PLRV isolate has been reported from India by Mukherjee et al. [14]. There is no information on complete genome sequences of Indian PLRV isolates. To check the level of variability, we have cloned and sequenced five PLRV isolates each one from North Western plains, Eastern plains, Northern high hills, North Eastern hills and Southern hills representing the different agroclimatic zones of India. We also report the comparative diversity analysis of these isolates with sixteen PLRV isolates reported previously from different countries and also with other leueoviruses.

Materials and Methods

Designing of Primers

Primers were designed using the complete genome sequences of PLRV isolates available in GenBank (AY138970, AF453392, AF453394, D13954 and D13953). The genome sequences were aligned using ClustalX software and two primer pairs were designed manually from the conserved regions and properties were checked by using online software, Oligo Calc: Oligonucleotide properties calculator (<http://www.basic.northwestern.edu/bio-tools/oligocalc.html>). These primer pairs amplify the genome of PLRV (5,883 nts) in two parts, 2,836 and 3,134 nts with a small overlapping region (Table 1). Sequencing primers were derived from the conserved regions of the genome sequences at every approximately 400 bp interval.

Virus isolates and Genome Amplification

The leaf samples of infected potato plants showing typical symptoms were collected from fields of five potato growing states of India viz., Jalandhar, Punjab (North Western plains); Kufri, Himachal Pradesh (Northern high hills); Patna, Bihar (Eastern plains); Ooty, Tamil Nadu (Southern hills) and Shillong, Meghalaya (North Eastern hills) during 2010–2011 and the isolates were designated as JPI-1, KHPI-1, PBI-6, OTNI-2 and SMI-5, respectively. The samples were tested through DAS-ELISA by following the standard protocol and RT-PCR using coat protein gene specific primers to ensure the PLRV infection before the amplification of genome. Total RNA was extracted from midrib and major veins of infected leaf using SpectrumTM Plant Total RNA kit (Sigma-Aldrich, Missouri, USA) and first strand cDNA was synthesized by using Revert aidTM First strand cDNA synthesis kit (Fermentas, Lithuania) with respective reverse primers according to the instructions of the manufacturer. PCR assays were carried out in a GeneAmp PCR system 9700 (Applied Biosystems). PCR programme consisted of pre-incubation at 95 °C for 3 min leading to 35 cycles of melting at 95 °C for 30 s, annealing at 64 °C for 30 s and synthesis at 68 °C for 3 min followed by an extension of 68 °C for 10 min. The RT-PCR products were analyzed by agarose gel electrophoresis in 1× TAE buffer (0.04 M Tris–acetate, 1 mM EDTA, pH 8) and the size of the products were identified by molecular weight markers (Fermentas, Lithuania).

Cloning and Sequencing

The amplified fragments were eluted using MinElute[®] gel extraction kit (Qiagen, Hilden, Germany) and ligated into pDrive cloning vector (Qiagen, Hilden, Germany) by following the instructions of the manufacturer. The ligation mixtures were used in transformation of *E. coli* strain JM107 (Fermentas, Lithuania) by heat shock method. The recombinant colonies were selected on LB agar supplemented with ampicillin (50 µg/ml) and IPTG-X-gal and confirmed by colony PCR analysis. Plasmid DNA was isolated from overnight grown culture using the

Table 1 Primers used for cloning of *Potato leafroll virus* genomes

S. no.	Primer	Sequence	Position	Amplicon size (bp)
1	PLRV-FP1	5'-ACAAAAGAATACCAGGGGAAATTGCAGC-3'	1–28	2,836
2	PLRV-RP1	5'-CAGCATCCAATACGCGACTGACC-3'	2,814–2,836	
3	PLRV-FP2	5'-GTGGAAGAATTGTGTGCAAATCAC-3'	2,750–2,773	3,134
4	PLRV-RP2	5'-ACTACACAACCCCTGTAAGAGGATCC-3'	5,859–5,883	

GenElute™ Plasmid Miniprep kit (Sigma-Aldrich, Missouri, USA). Restriction digestion analysis was performed with *KpnI* enzyme (New England Biolabs, USA) to linearize the recombinant plasmid and molecular weight markers (Fermentas, Lithuania) were used to check the size.

Sequencing of the cloned PCR products was done using the vector-encoded M13 forward primer (−20), M13 reverse primer and twenty internal primers. Two independent clones for each isolate were sequenced using the automated DNA sequencer, ABI PRISM™ 310 Genetic Analyzer (Applied Biosystems). Sequencing reactions were performed in Gene Amp PCR system 9700 (Applied Biosystems) with BigDye terminator kit 3.1 according to the manufacturer's instructions (Applied Biosystems). Sequencing products were purified as per manufacturer's instructions using ethanol precipitation prior to resuspension in formamide. The sequences were assembled using DNAbaser to form contigs, the peaks were checked and manually edited. The sequences were submitted in GenBank and the accession numbers are JQ420901–JQ420905.

Phylogenetic Analysis

Multiple sequence alignments were made and per cent similarity was calculated by using Bioedit software. Phylogenetic trees were constructed and a bootstrapped consensus dendrogram was generated with 1,000 replications using Neighbour joining algorithm of MEGA software version 4.0.2 [23]. Complete genome sequences of sixteen PLRV isolates were used for comparative genome analysis. The nucleotide sequences of the Indian isolates were examined for the recombination events using the recombination detection method SISCAN of RDP3 software [5, 11]. The other luteovirus sequences used for comparative studies are as follows: *Pepper vein yellows virus* (PeVYV; NC_015050, AB594828), *Barley yellow dwarf virus-PAV* (BYDV-PAV; D85783), *Cereal yellow dwarf virus—RPV* (CYDV-RPV; L25299), *Tobacco vein distorting virus* (TVDV; NC_010732, EF529624), *Cucurbit aphid-borne yellows virus* (CABYV; GQ221224, GQ221223), *Beet western yellows virus* (BWYV; EU636991), *Melon aphid-borne yellows virus* (MABYV; NC_010809), *Beet mild yellowing virus* (BMYV; DQ132996), *Chickpea chlorotic stunt virus* (CpCSV; JF507725), *Sugarcane yellow leaf virus* (SuYLV; GU190159) and *Turnip yellows virus* (TuYV; NC_003743). The sequence identity of different ORFs at both nucleotide and amino acid levels between the Indian isolates were predicted. The deduced amino acid sequences of ORF 0, ORF 2, ORF 3 and ORF 4 of Indian isolates were also compared with the available sequence information.

Results

Genome Sequences of PLRV Isolates

The genome of PLRV isolates was 5,883 nucleotides long and had nine predicted open reading frames (ORF 0 to ORF 8) that were similar to the other PLRV isolates. Except the isolate OTNI-2 in which a single nucleotide substitution (A>G) in the stop codon (at position 5,742) of ORFs 5 and 7 was observed. The genome was predicted to contain a non coding sequence of 70 nucleotides at the 5' end, 144 nucleotides at the 3' end and 197 nucleotides in between the two blocks of coding sequences. About 97.6–98.7 % similarity was observed within the Indian isolates. The nucleotide sequence identities for the individual ORFs of Indian PLRV isolates were 94.3–99.4 % (ORF 0), 97.0–99.2 % (ORF 1), 98.0–99.0 % (ORF 2), 98.8–99.8 % (ORF 3), 98.9–99.7 % (ORF 4), 97.6–98.3 % (ORF 5), 96.8–99.4 % (ORF 6), 95.5–97.3 % (ORF 7) and 97.1–100 % (ORF 8). At nucleotide level, ORF 3 and ORF 4 are the most conserved ones. The isolates showed more divergence in the ORF 0 region especially the isolate, PBI-6 was different from other Indian isolates and had only 94.3–95.1 % similarity to other four isolates.

Comparison with Other PLRV Isolates and Luteoviruses

Nucleotide sequence comparison with sixteen PLRV sequences reported from different countries indicated that these isolates shared 95.8–98.6 % similarity to European, Canadian, American, African, Czech isolates and a minimum of 92.9–93.4 % similarity to an Australian isolate (D13953). All the five isolates showed maximum similarity to Poland and Egyptian isolates. The isolates JPI-1, KHPI-1, PBI-6, OTNI-2 and SMI-5 were 98.6, 98.5, 97.9, 98.5 and 98.4 and 98.4 and 98.3 % similar to the Poland (X74789) and Egyptian (AY1389970) isolates, respectively. The phylogenetic tree based on the genome sequence allowed the distribution of isolates into two major groups (Fig. 1). Group 1 contained the isolates from Czech Republic, USA, Canada, Europe, and Africa in which the Indian isolates were also grouped. Whereas the Australian isolate was found in group 2. Recombination analysis using SISCAN method showed that the Indian isolate, PBI-6 is a recombinant isolate derived from a major parent, EU717546 (Czech isolate) and a minor parent, AF453389 (Spain isolate) and the beginning point was undetermined and ended at 585 nucleotide with a average SISCAN p value of 8.016×10^{-12} . Maximum of 3 recombination events were predicted.

In addition, the isolates were also compared with the other members of the genus *Potavirus*. Nucleotide

Fig. 1 Phylogenetic tree generated based on the complete genome sequence of Indian PLRV isolates and other previously reported PLRV isolates from GenBank. The bootstrap analysis was performed by the NJ method and drawn using MEGA 4.0 software. The scale bar represents a genetic distance of 0.005. (The isolates of the present study are highlighted with black diamond)

identities of these isolates with other poleroviruses ranged from 43.7 to 53.1 % with a maximum of 52.8–53.1 % similarity to CYDV. The identities ranged from 45.3–45.6 % to CABYV, 45.1–45.2 % to MABYV, 46.6–46.9 % to BWYV, 46.4–46.5 % to BMV, 46.8–47.0 % to TuYV, 44.2–44.3 % to PeVYV, 46.5–46.6 % to TVDV, 43.7–44.0 % to ScYLV and 44.1–44.3 % to CpCSV. The isolates shared 29.1–29.3 % similarity to BYDV which belongs to another genus *Luteovirus* of the family *Luteoviridae*.

Analysis of Predicted Proteins

The amino acid sequence identities for the individual ORFs of Indian PLRV isolates were 93.1–99.1 % (ORF 0), 94.9–98.1 % (ORF 1), 97.8–98.8 % (ORF 2), 98–100 % (ORF 3), 97.4–99.3 % (ORF 4), 96.9–97.4 % (ORF 5), 93.5–100 % (ORF 6), 91.2–94.4 (ORF 7) and 97.8–100 % (ORF 8). In ORF 0 region, at amino acid level also the isolate, PBI-6 showed only 93.1–93.5 % similarity to other four isolates.

The identity with the other previously reported PLRV isolates for P0 ranged between 85.1 and 100 %, for RdRp

ranged between 96.2 and 99.6 %, for coat protein ranged between 96.6 and 100 % and for movement protein it is 93.5–100 %. Phylogenetic analysis based on the amino acid sequence of ORF 2, ORF 3 and ORF 4 regions showed that the Indian isolates were grouped with Czech, France, Poland and Cuba isolates. Phylogenetic tree based on the amino acid sequence of ORF 0 found to form two groups. The isolates, JPI-1, KHPI-1, OTNI-2 and SMI-5 were grouped along with the isolates from different countries. Whereas the isolate, PB-6 was found in the second group along with Peru (AF453392) and Czech (EU717545) isolates.

Amino acid changes specific to Indian isolates were observed (Table 2). More number of amino acid changes was observed in ORF 2 than in ORF 3, ORF 4 and ORF 0. In the ORF 2 region, six amino acid changes were observed in each of the isolates JPI-1, OTNI-2 and SMI-5 and two amino acid changes in KHP-1 and PBI-6. In ORF 3, only two amino acid changes in the isolates JPI-1 and SMI-5 and in ORF 4 a single amino acid change in JPI-1 and KHPI-1 was observed. In ORF 0, a single amino acid change in each of the isolates, JPI-1, KHPI-1 and PBI-6 was observed.

Table 2 Amino acid changes specific to Indian *Potato leafroll virus* isolates

ORFs	Protein	JPI-1	KHPI-1	PBI-6	OTNI-2	SMI-5
ORF 0	P0	L59P	R118G	V100L	-	-
ORF 2	RNA-dependent RNA polymerase	F31S, R110Q, N195S, A208V, A389S, E598G	E94K, C460R	F31S, T66A	E116D, Y502H, C504S, T529S, K533N, A601V	K64R, E103K, T106A, K119I, A389V, P402L
ORF 3	Coat protein	G53S, T192I	-	-	-	V14I, L204M
ORF 4	Movement protein	Y6F	A131V	-	-	-

In the coat protein region, the isolates OTNI-2, KHPI-1 and PBI-6 were 100 % similar to each other. The five isolates shared 98–100 % similarity at amino acid level with the coat protein sequence of a severe strain of PLRV which was reported earlier from our institute (AF539791) and with another isolate from India (GU256062). Whereas, the coat protein shared only 38.2–63.6 % similarity to other poleroviruses with a maximum similarity to BWYV and minimum similarity to SuYLV.

Discussion

The genome sequences of five PLRV isolates were obtained and compared with the sixteen sequences available in GenBank. The genome organization was similar to other PLRV isolates except in one isolate, OTNI-2 in which a single nucleotide substitution (A>G) in the stop codon (at position 5,742) of ORFs 5 and 7 was observed which may leads to extension of these proteins. But we donot have any evidence for the extension of the proteins. Nevertheless, a similar substitution at the stop codon (UGA>CGA) of ORF 0 of the isolate 14.2 and extension of 9 % of the protein by translation of 22 extra amino acids at the C terminus of P0 before next in-frame stop codon was reported by Guyader and Ducray [8].

Identity among these five isolates ranged from 97.6 to 98.7 % and this shows that the level of sequence variation among Indian PLRV isolates is low as observed in the sixteen PLRV isolates reported so far except one isolate from Australia. Earlier reports clearly indicate that all the isolates viz., Canada, Netherland, Scotland [10], Poland [15], Spain, France, Peru, Cuba, Zimbabwe [8], Egypt [2] and Czech Republic [19] are closely related to each other than Australian isolate. The Australian isolate reported to be the most divergent among the PLRV isolates particularly by about 600 nt in the polymerase-coding region and has an nucleotide identity of 93 % with other isolates [10]. Our study shows that the Indian isolates were also similar to the former group than the Australian isolate. The Indian isolates showed maximum similarity to Poland (X74789) and Egyptian (AY138970) isolates and the reason for this may be that the potato was introduced from Europe to India and also the germplasms are being imported from the northern hemisphere countries for breeding purpose. In a comparison with other poleroviruses, the Indian isolates shared a maximum of 52.8–53.1 % similarities to CYDV and formed a separate group with CYDV and the earlier report [8] also shows that CYDV is closely related to PLRV.

Recently, Zarghani et al. [25] reported the formation of two stable groups of all PLRV isolates based on the amino acid sequence of ORF 0 and they also reported that the recombination may increase diversity in ORF 0 region. In

support to their findings, the isolate, PBI-6 was divergent from other four isolates in ORF 0 region and found to form a separate group which was predicted to be a recombinant between Czech (EU717546) and Spain (AF453389) isolates. Specific amino acid changes were observed in Indian isolates. Such changes may be introduced by error prone RNA-dependent RNA polymerase during the replication process [3]. The changes were comparatively more and found in all the five isolates (six in JPI-1, OTNI-2 and SMI-5, two in KHP-1 and PBI-6) in ORF 2 region. In the other two ORFs (ORF 3 and ORF 4), only one or two amino acid changes in two of the five isolates were observed. According to Guyader and Ducray [8] and Plchova et al. [19], ORF 3 and ORF 4 are the most conserved regions in PLRV and our study also shows that these regions are >98 % similar within the Indian isolates. The isolates also shared 98–100 % similarity at amino acid level with the coat protein of two PLRV isolates reported earlier from India.

To our knowledge, this is the first report of complete genome sequence of PLRV isolates from different agro climatic zones of India. Although mutations and recombination events (as in the isolate PBI-6) were detected, the Indian isolates in the present study showed low level of genetic diversity.

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