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The complete nucleotide sequence of Passiflora latent virus and its phylogenetic relationship to other carlaviruses*

Brief Report

S. Spiegel[1], **M. Zeidan**[2], **I. Sobolev**[1], **Y. Beckelman**[1], **V. Holdengreber**[1], **Y. Tam**[1], **M. Bar Joseph**[1], **Z. Lipsker**[1], and **A. Gera**[1]

[1]Department of Plant Pathology, Agricultural Research Organization, The Volcani Center, Bet Dagan 50250, Israel
[2]The Plant Protection and Inspection Services, Ministry of Agriculture, Bet Dagan 50250, Israel

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Summary. A virus identified as Passiflora latent virus (PLV) was isolated from passion fruit plants. Particle morphology, host range and serological properties suggested that this virus belongs to the genus *Carlavirus*. The complete genomic sequence of PLV was determined by sequencing overlapping cDNA fragments. The genome consisted of 8386 nt, excluding the poly (A) tail and contained six open reading frames, typical of carlaviruses. The overall similarities of the predicted amino acid sequence of PLV to those of other carlaviruses ranged from 25 to 73%. Phylogenetic analysis indicated that PLV was closely related to lily symptomless virus and blueberry scorch virus. This is the first report of the complete nucleotide sequence and genome structure of PLV.

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Passiflora edulis, native to South America, is one of the few species cultivated for its edible tropical fruit, known by the popular name passion fruit. Cultivars of *P. edulis* f. *edulis* Sims, bearing aromatic purple fruit and *P. edulis* Sims f. *flavicarpa* Degener, bearing yellow fruit are increasingly being appreciated as fresh or processed fruit in areas out of their natural habitat and are grown commercially in tropical and subtropical parts of the world [21].

Worldwide, traditional propagation of passion fruit plants has been from seeds, resulting inevitably in a diverse seedling population. Mass production of

*The nucleotide sequence reported in this manuscript has been deposited in GenBank under the accession number DQ455582.

genetically uniform planting material, desired by growers, has been accomplished through vegetative propagation, namely, rooting cuttings and/or grafting. Like other vegetatively propagated plants, transmission of pathogens, mainly viruses, in nursery stock plants is a potential problem.

Viral diseases have been reported both in purple and yellow passion fruit. A partial list includes a potyvirus (passion fruit woodiness virus [30]), a rhabdovirus (passion fruit vein clearing virus [19]), a tymovirus (passion fruit yellow mosaic virus [11]) and a cucumovirus (cucumber mosaic virus [31]). Passiflora latent virus (PLV), genus *Carlavirus* [7], has been reported to occur naturally in *Passiflora* spp. in Germany, Australia and USA [6, 23, 29]. PLV virions were slightly flexuous rods with a length of 650 nm [5, 23]. The experimental host range of PLV was very narrow and limited to *Chenopodium murale* and *C. quinoa* [5].

Unlike other viruses in the genus *Carlavirus* e.g. lily symptomless virus (LSV), garlic latent virus (GLV), potato virus M (PVM), and others for which partial or complete genome nucleotide sequences have been published [8, 9, 32], no

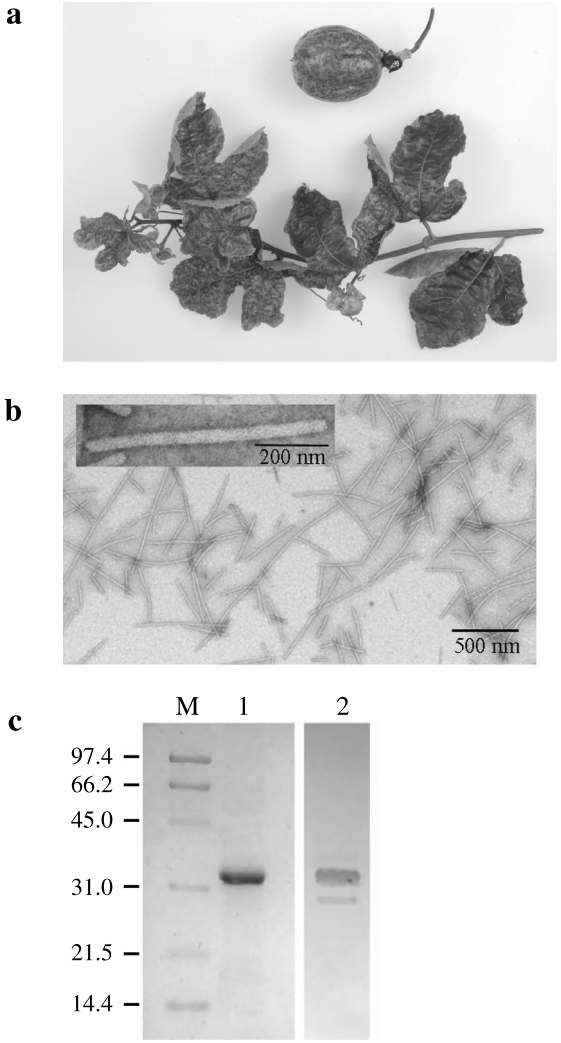

Fig. 1. a Leaf and fruit symptoms on passion fruit plants naturally infected with PLV. **b** Electron micrograph of PLV particles stained with uranyl acetate and decorated with specific antiserum (insert). **c** SDS–polyacrylamide gel electrophoresis of isolated PLV stained with Coomassie brilliant blue (lane *1*), immunoblot probed with PLV polyclonal antibodies (diluted 1:1000) (lane *2*)

molecular information has been reported for PLV. Here, we report the complete nucleotide sequence of the genome of PLV, a distinct carlavirus isolated from purple passion fruit in Israel. The elucidation of its relatedness to other viruses of the same genus is included.

The source plant material used in this study was a vine of purple passion fruit (*P. edulis* f. *edulis* Sims) cultivar 'Passion Dream' displaying foliar and fruit mosaic symptoms (Fig. 1a). Symptoms were pronounced in the spring and virtually disappeared during the summer. The plant was transferred from a commercial field plot to a screen house at the Volcani Center.

For determination of the experimental host range, mechanical transmission of the virus was attempted by grinding leaves from the symptomatic passion fruit vine in 1% K_2HPO_4, and inoculating 21 different herbaceous indicator plants (not listed in this report) and healthy passion fruit seedlings. Inoculated plants were maintained at 22–24 °C for up to 30 days for symptom development. The virus was transmitted only to *C. murale* and *C. quinoa* and to passion fruit seedlings. Symptoms included local lesions on inoculated leaves followed by systemic necrosis and mottling on upper leaves. On passion fruit seedlings, conspicuous mosaic symptoms were observed on inoculated leaves. None of the other tested herbaceous plants showed symptoms during the post-inoculation period. Plants were tested for PLV by ELISA [23], 20–30 days post inoculation, and the obtained results supported the above data. Attempts to transmit the virus from the infected *Chenopodium* spp. to other test plants failed. Similar results were previously reported for PLV [6, 29].

Flexuous, elongated virus particles with a modal length of about 660 nm were consistently observed in negatively stained leaf dip preparations from infected passion fruit, *C. murale* and *C. quinoa* plants (not shown). Electron microscopy (EM) studies of ultrathin sections of infected passion fruit tissue revealed the presence of elongated rods resembling virus particles in the mesophyll and vascular cytoplasm of parenchyma but not in the nucleus. No pinwheels, typical of potyviruses, have been observed and no cytopathological effects were noticed (not shown).

The virus was purified from inoculated, symptom-expressing *C. quinoa* plants [10] and was used to prepare polyclonal antibodies [14]. Preparations of purified virus for EM contained numerous flexuous elongated particles (Fig. 1b), which gave a strong decoration reaction with the specific antiserum produced against this PLV isolate (Fig. 1b, inset). No reaction was obtained with antibodies against LSV and PVM.

Electrophoresis of SDS-disrupted purified virus preparations revealed a major polypeptide band with an estimated Mr of 32 kDa (Fig. 1c, lane 1). This value is within the range (31–34 kDa) listed for carlaviruses [13]. Immunoblots with the PLV antiserum gave a strong, clear reaction with the polypeptide corresponding to the coat protein (Fig. 1c, lane 2). No reactions were obtained with antisera against LSV and PVM (not shown).

The nucleotide sequence of the complete genome of PLV was determined. Total RNA was extracted from a PLV-infected passion fruit plant and from

symptomatic, infected *C. quinoa* [28]. Double-stranded (ds) RNA was isolated from the infected passion fruit plant [22]. The purified RNA preparations were resuspended in RNase-free water and used for cDNA synthesis.

For cloning of the PLV genome, reverse transcription (RT) was carried out using Superscript III (Invitrogen, USA) following the supplier's instructions. Amplification of PLV fragments was done by PCR, either using Hot Start AccuPower premix for short fragments or AccuPower HL PCR premix for long fragments, according to the supplier's instructions (Bioneer, Korea).

Initially, amplified products consisting of a 136-bp fragment from the 3′ end of the viral genome and a 280-bp product from the 3′ end of the replicase were generated by RT-PCR using an oligo (dT) and the Carla-Uni primer [27], and a carlavirus group PCR primer mix (93000/0025, Agdia, USA), respectively. Amplified products were cloned into pGEM-T Easy Vector (Promega, USA) and the nucleotide sequence of each insert was determined using an automated ABI Prism 3700 DNA analyzer (Hy Laboratories, Israel). Based on the acquired sequence data, primers PLVR8250-8223 and PLVF5518-5543 were designed to generate a cDNA fragment that would bridge the gap between the viral 3′ and replicase regions described above. PCR products were processed (as above) and recombinant plasmids with PLV-derived cDNA inserts were sequenced to compile and determine the nucleotide sequence of the viral genome. Each region of the genome was covered by at least two overlapping clones.

To obtain the PLV genome fragment corresponding to the 5′-terminal region (upstream of the 280-bp fragment), two cloning strategies were used: in the first, 3′-dsRNA tailed products were subjected to RT-PCR following Attouii et al. [3] with minor modifications. A 3′-amino blocked and 5′-phosphorylated oligonucleotide #1, 5′-PO4-AGGTCTCGTAGACCGTGCACC-NH2-3′, was ligated to dsRNA obtained from PLV-infected plants by using T4 RNA ligase (BioLabs, USA). The tailed dsRNA was recovered from the ligation mixture [26], and RT-PCR was subsequently carried out using primer PLVR5295-5268 and oligonucleotide #2, 5′-GGTGCACGGTCTACGAGACCT-3′ [3]. Sequencing of three overlapping clones revealed the sequence of the major part of ORF1. A second strategy based on the SMART-TM method with Capswitch oligonucleotide and a specific PLV primer was employed to obtain the remaining 5′- terminal region. The ligation step was identical to the dsRNA tailing step (described above) except for the use of the Capswitch oligonucleotide, 5′-AAGCAGTGGTATCAACGCAGAGTGGCATT ACGGCCGGG-3′ (Clontech, USA). RT-PCR was carried out with PLVR2712-2688 and SMART forward primer, 5′-AAGCAGTGGTATCAACGCAGAGT-3′, using reagents from the SMART cDNA cloning kit (Clontech). The sequence revealed the remaining nucleotide sequence of the 5′- viral terminus and the 5′-part of ORF1. Specific primers targeting various regions were used to ascertain the sequence of the PLV genome (data not shown).

Assembly of the complete nucleotide sequence of PLV was done by using DNAMAN DNA analysis software (Lynnon Biosoft, 1994) based on the sequences of three independent clones in both directions for each fragment. Database searches were done using BLAST [2], and pairwise alignments of

nucleotide and amino acids sequences were done using BioEdit version 7.0.5.2 [16]. Multiple alignments of amino acid sequences were done using CLUSTAL X [18] embedded in the BioEdit program. Phylogenetic analysis was performed to determine the relationship of PLV with other carlaviruses by using the neighbor-joining algorithm [25] with 1000 bootstrap replicates. Trees were viewed using the NJplot program [24]. The following carlaviruses (and their accession numbers) were used in sequence alignments and phylogenetic analysis: Aconitum latent virus (AcLV), AB051848; blueberry scorch virus (BlSV), L25658; garlic strain of shallot latent virus (GLV), AJ292226; hop latent virus (HpLV), AB032469; lily symptomless virus (LSV), AJ516059; potato virus M (PVM), D14449; potato virus S (PVS), AJ863509; poplar mosaic virus (PopMV), AY505475; sweet potato chlorotic fleck virus (SPCFV), AY461421.

The complete genome of PLV was 8386 nucleotides (nt) long excluding the poly (A) tail at the 3′ terminus (GenBank accession no. DQ455582). The overall identities between PLV and nine carlaviruses, for which complete nucleotide sequence data are available, were 65, 64, and 63% with LSV, BlSV, and PVS, respectively, and 59–56% with six others (not shown). The PLV genome contains six ORFs with an arrangement typical of carlaviruses [1, 12]. The 5′ and 3′ untranslated regions of the PLV genome were 71 and 51 nt, respectively. ORF1 encodes the predicted helicase/replicase proteins present in carlaviruses, while ORFs 2–4 encode the triple gene block (TGB). ORF5 encodes the coat protein (CP) and ORF6 a putative nucleic-acid-binding protein. The calculated similarities between each of the PLV ORFs to those of other carlaviruses are presented in Table 1.

PLV ORF1 (nt 72–5912) is predicted to encode a polypeptide of 1946 aa with a calculated Mr of 219 kDa and shares common features with ORF1-encoded proteins of other carlaviruses. It contains motifs for a type 1 methyltransferase (MTR1) in its 5′ part and a putative NTP-binding/helicase including

Table 1. Comparison of nucleotide (nt) and amino acid (aa) sequence identities (percentage, %) in the individual open reading frames (ORF) between PLV and other carlaviruses

Virus[a]	ORF1		ORF2		ORF3		ORF4		ORF5		ORF6	
	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa
LSV	**64**	**60**[b]	63	62	**69**	**67**	59	48	**73**	**69**	65	49
BlSV	63	56	**65**	**63**	66	65	**61**	**52**	69	67	**60**	**51**
PVS	62	51	63	61	67	60	56	29	68	67	57	47
AcLV	59	45	60	55	64	52	57	36	59	49	57	42
HpLV	60	48	62	53	58	51	56	39	58	48	57	43
PVM	60	44	60	52	62	54	56	31	57	45	62	41
PopMV	59	44	57	39	59	40	53	31	56	34	57	34
GLV	76	44	54	35	58	49	55	29	57	38	54	39
SPCFV	57	41	56	36	60	43	53	25	57	38	52	20

[a]Abbreviations of virus isolates and the accession numbers of their nucleotide sequences are provided in the text

[b] Bold characters indicate the highest % identities between PLV and other carlaviruses

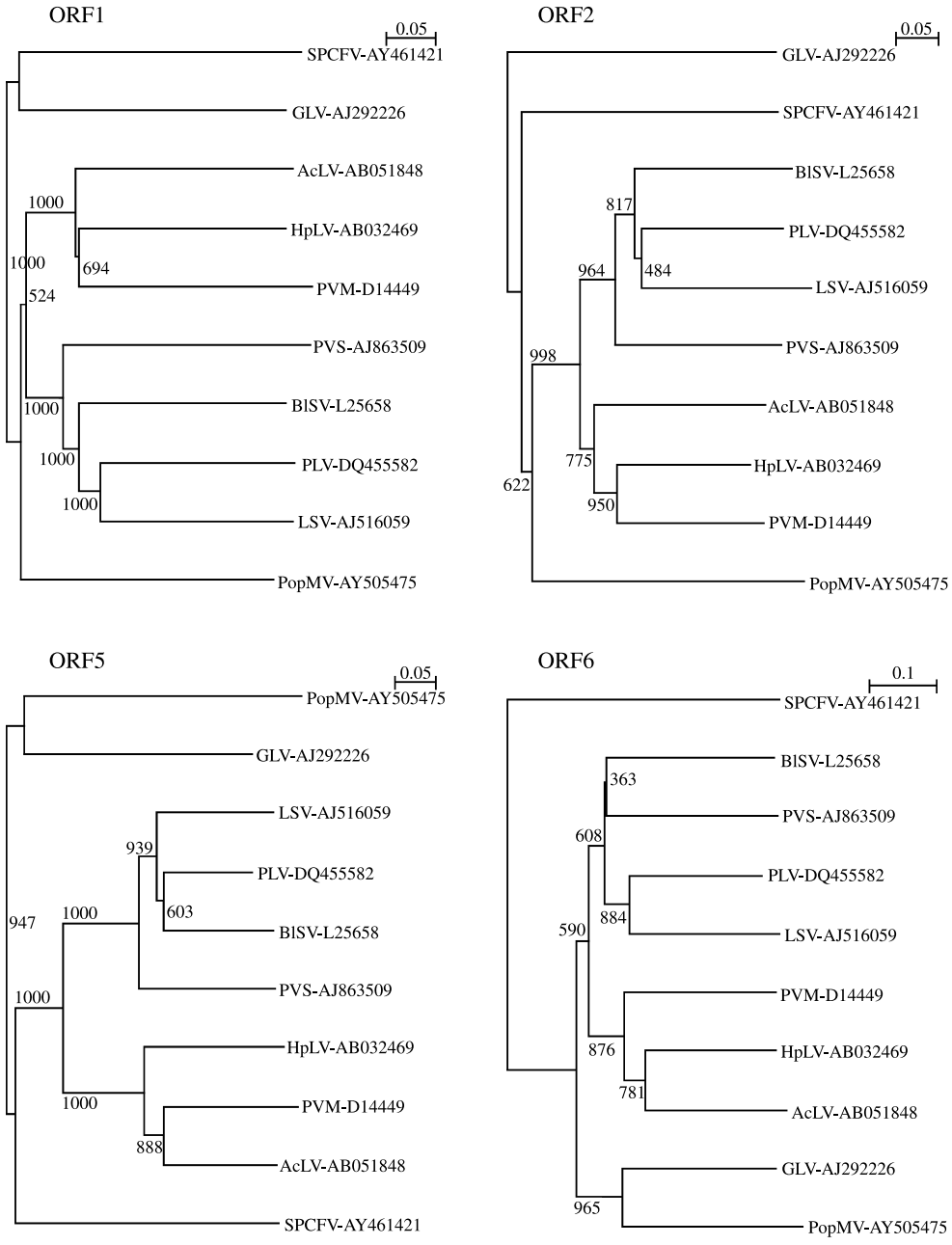

Fig. 2. Phylogenetic analysis of the amino acid sequences of ORF1, ORF2, ORF5, and ORF6 of PLV and other carlaviruses. Sequences were aligned using CLUSTALX with gap-opening and extension penalties of 10.0 and 0.2, respectively, and Gonnet series protein weight matrix. Phylogenetic trees were inferred using the PMB model and neighbor-joining method with 1000 bootstrap replicates using Phylip within BioEdit program version 7.0.5.2. Trees were viewed using the NJ plot program. Abbreviated virus names are identified in full in the text

G1170X4G1175K1176S1177, representative of the superfamily1 group [20], in its central region. The known motifs corresponding to viral RNA-dependent RNA polymerases (RdRp) were found in the C-terminal region of ORF1. A putative G1843D1844D1845 (GDD) motif, residing within the core of RdRp, was also found. The GDD motif, involved in RNA replication, is conserved across most positive-stranded RNA viruses. PLV ORF1 was closest to LSV and most distant from SPCFV (Table 1 and Fig. 2 ORF1).

PLV ORF2 (nt 5940–6629), separated from ORF1 by an intergenic region of 27 nt, is predicted to encode a protein of 229 aa with a calculated Mr of 25.2 kDa. ORF2 contains a NTP-binding/helicase motif (G33K34S35) in the N-terminal region. This motif is highly conserved in NTP-dependent DNA helicases [15]. Together, ORF2, ORF3 (nt 6607–6930) and ORF4 (nt 6909–7103) encode polypeptides of 229, 107 and 64 aa with calculated Mr of 25, 11.5 and 7 kD, respectively, known as TGB. Like other carlaviruses, ORF2 overlaps with ORF3, which overlaps with ORF4. This organization of TGB is conserved among carlaviruses and potexviruses [4]. Both ORF2 and ORF4 of PLV share high similarities to BlSV, whereas ORF3 is closest to LSV (Table 1). However, phylogenetic analysis indicates that PLV ORF2 is more closely related to BlSV (Fig. 2 ORF2), and for ORF3 and ORF4, no significant results, as indicated by the bootstrap values, were obtained (not shown).

PLV ORF5, encoding the CP, is separated from ORF4 by a second intergenic region (nt 7104–7144). In our data, ORF5 has two potential initiation codons, the first starting at nt 7145 and the second at nt 7202. Based on the immunoblot results (Fig. 1c), we concluded that the first initiation codon at nt 7145 constitutes the start of the CP gene and nt 8005 its end. Thus, ORF5 encodes the PLV CP consisting of 286 aa with a calculated Mr of 31.7 kDa. This value is within the range listed for carlaviruses [13]. ORF5 has the highest aa sequence identity (69%) with LSV and shares 67–34% identity with other carlaviruses. The similarity to LSV represents the highest value obtained for all ORFs (Table 1, Fig. 2 ORF5). Previous studies have shown that a high similarity of predicted amino acids in the CP region of carlaviruses was correlated with their serological properties [17]. An alignment of the CP amino acid sequences of PLV and LSV reveals a high identity over the entire CP except for the N-terminal region (not shown). Considering the molecular demarcation criteria according to which members of individual carlavirus species have less than ca. 72% identical nt or 80% identical aa between their entire CP or replication protein genes [1], the genomic characteristics of PLV support its classification as a distinct carlavirus.

PLV ORF6 (nt 7989–8333) encodes a polypeptide of 114 aa with a calculated Mr of 13 kDa. The size of this potential NABP protein is relatively small among the carlaviruses. ORF6 contains a zinc finger metal binding motif ($C_{58}X_2C_{61}X_{12}C_{74}X_4C_{78}$) in the C-terminal region. It showed 51 and 49% identity with ORF6 of BlSV and LSV, respectively (Table 1). However, the phylogenetic tree analysis of ORF6 supports a similarity between PLV and LSV (Fig. 2 ORF6).

This is the first report of the complete nucleotide sequence and genome structure of PLV. The results of bioassays and serological and EM studies presented

in this paper are in agreement with previously reported results indicating that PLV is a carlavirus. The molecular data in this report confirm that PLV shares the major functional motifs and sequences that are conserved with other members of this group.

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Author's address: Dr. S. Spiegel, Department of Plant Pathology, ARO, The Volcani Center, Bet Dagan 50250, Israel; e-mail: spiegels@volcani.agri.gov.il