BIOLOGICAL ACTIVITY OF TRANSCRIPTS FROM cDNA OF PELARGONIUM LINE PATTERN VIRUS

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Received June 20, 2007; accepted October 22, 2007

Summary. – A set of cDNAs of Pelargonium line pattern virus (PLPV) was assembled under the control of T7 RNA polymerase promoter and ligated into the plasmid pUC18. Transcripts synthesized *in vitro* from cDNA were infectious on *Chenopodium quinoa* according to locally induced lesions and hybridization assay. The biological activity of the viral transcripts was particularly sensitive to the short 3' terminus extensions, whereas inclusion of the 3 extra bases at the 5' terminus did not substantially affect the infectivity. Inoculation of the transcripts on plants *Nicotiana benthamiana* and *Nicotiana clevelandii* give rise to the systemic infection indistinguishable from that established by the parental isolate. This is the first report about the preparation of infectious RNA transcripts from a full-length cDNA clone of PLPV.

Key words: Pelargonium line pattern virus; cDNA; infectious transcripts

PLPV is one of the most frequent viral pathogens in *Pelargonium* spp. worldwide (Stone, 1980; Bouwen and Maat, 1992; Franck and Loebenstein, 1994; Alonso and Borja, 2005). The infections are often asymptomatic, but under certain environmental conditions, the yellow-green spots and line patterns on the leaves may appear what impair their quality and marketability (Nameth and Adkins, 1993). It should be noted that PLPV is proposed as a tentative member of a prospective new genus in the family *Tombusviridae* (Kinard and Jordan, 2002; Castaño and Hernández, 2005) though the virus has not been formally classified into taxonomic scheme yet (Fauquet *et al*., 2005). This is most likely due to the limited information on PLPV and other related viruses infecting geranium, what has complicated their classification to a viral group so far.

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Abbreviations: cDNA = complementary DNA; dsRNA = double-stranded RNA; gRNA = genomic RNA; PLPV = Pelargonium line pattern virus; RT = reverse transcription; ssRNA = single-stranded RNA

PLPV virions are isometric, about 30 nm in diameter and contain a linear positive-sense single-stranded RNA (ssRNA). The complete nucleotide sequence of PLPV genomic RNA (gRNA) has been recently determined (Castaño and Hernández, 2005). It comprises 3884 nt and contains 6 ORFs flanked by an unusually short untranslated regions of 6 nt at the 5' end and 247 nt at the 3' end. The 6 ORFs potentially encode proteins of 27 (p27), 13 (p13), 87 (p87), 7 (p7), 6 (p6) and 37 (p37) K. The ORF arrangement on the PLPV genome closely resembles the one found in the members of genus *Carmovirus*, family *Tombusviridae*. Moreover, the putative PLPV gene products presumably involved in replication (p27 and its readthrough product p87), movement (p7), and encapsidation (p37) show a high identity with proteins of this viral group. However, some unique characteristics distinguish PLPV from the carmoviruses, as the production of only one subgenomic RNA in contrast to the carmoviruses that generate two. Three related small isometric ssRNA viruses, Pelargonium ringspot virus (unclassified), Pelargonium chlorotic ring pattern virus (unclassified), and Elderberry latent virus (a tentative species of the genus *Carmovirus*) share the same characteristics. In advance, it has been suggested that they should be grouped

together with PLPV into a new genus *Pelarspovirus* within the family *Tombusviridae* (Kinard and Jordan, 2002; Castaño and Hernández, 2005).

The availability of a full-length cDNA prepared from the viral genome capable of providing infectious transcripts has enormously contributed to the studies about genomic functions of plant viruses. In this paper, we report the construction of a full-length cDNA of the PLPV genome under the control of the bacteriophage T7 promoter that served as a template for the synthesis of infectious transcripts *in vitro*. To our knowledge, this is the first report about the preparation of cDNA clone for a tentative member of the prospective genus *Pelarspovirus*. We supposed that preparation of cDNA allowed us to perform studies concerning the replication, gene expression and pathogenesis of this viral group.

PLPV, isolate PV-0193, was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) and maintained by a sap inoculation on the experimental host *Chenopodium quinoa*. Total RNA from infected *C. quinoa* leaves was prepared by the phenol extraction and lithium chloride precipitation (Verwoerd *et al.*, 1989) and used as a template for RT-PCR reaction with Superscript II-RT (Invitrogen) and Expand High Fidelity DNA polymerase (Roche). Two overlapping cDNAs were generated, one with primers CH18 (5'-GCCTCCCTGCTGGCATAAACTAATAC-3') complementary to nt 3578–3603 of PLPV gRNA and CH61 (5'-GCAAGCTTGTAATACGACTCACTATAGGGAACA AAATGGCACACTATTTTGG-3'), which contains a *Hind*III site (underlined) fused to a T7 RNA polymerase promoter sequence (in bold) followed by 23 nt of the 5' end of the virus sequence. The second cDNA was generated with primers CH17 (5'-GAAAATGGCCTTCTACGGGGAC-3'), homologous to nt 2067–2088, and CH60 (5'-CC*GG ATCCCGGG*CAGATCAGGGGGGTGGGTTAC-3'), complementary to the 3´ terminus of the viral sequence (nt 3859–3884) with a *Sma*I site (underlined) and a *Bam*HI site (in italics) at the 5' terminus. Ligation of the RT-PCR products through the *Xmn*I site (present in the PLPV sequence at nt 3091–3100) allowed us to generate independent full-length viral cDNAs, which were subsequently cloned into *Hind*III and *Bam*HI sites of the plasmid pUC18. Two recombinant plasmids were prepared and signed as pPLDS-1 and pPLDS-2. The plasmids were digested with *Bam*HI and transcribed using T7 RNA polymerase (Fermentas). No cap analog was included in the transcription reactions as the cap structure is not required for infectivity of viral RNA among the members of the family *Tombusviridae*. After recognition of its promoter, T7 RNA polymerase will start transcription at the first of the 3 terminal G nt in the promoter sequence, thus the transcripts were expected to contain 3 extra G nt at the 5' terminus compared to the viral RNA. In addition, as the plasmids were linearized with *Bam*HI prior *in vitro* transcription, the transcripts were also predicted to harbor 5 extraviral nt (GGAUC) at the 3' terminus derived from the *Bam*HI target site. The *C. quinoa* plants were mechanically inoculated with RNAs derived from each cDNA clone (approximately 0.7 µg per leaf). The mock-inoculated plant served as a negative control. None of the inoculated leaves developed chlorotic lesions typical of PLPV infections. Likewise, hybridization assays confirmed the absence of the virus in the inoculated leaves. To study more closely the absence of infectivity of these clones, we determined their nucleotide sequences that showed 99% nt identity with the PLPV (Castaño and Hernández, 2005). No obvious lethal nucleotide changes were detected in the construct pPLDS-2. On the other hand, one of the nucleotide substitutions present in the construct pPLDS-1 (C703T) led to the introduction of a premature stop codon in ORF (p27), which might be responsible for the non-viability of the corresponding transcripts.

Two following sets of partial cDNAs were generated by RT-PCR using two different pairs of primers: CH61 and CH64 (5'-CCAGTCTAGACCTCACGCCAATTTC-3'), complementary to the nt 1663–1687 of the PLPV gRNA, or CH60 and CH63 (5'-CGTGAGGTCTAGACTGGTGA ACAACG-3'), homologous to the nt 1671–1697. These cDNAs covering the 5' and 3' portion of the gRNA, respectively, and containing an *Xba*I site (present at nt 1678–1683 in PLPV genome) in the overlapping region, were used to ready exchange of these regions in the initial non-infectious construct pPLDS-2. Transcripts derived from several of the resulting clones with the 5' portion replaced, induced chlorotic lesions when inoculated on *C. quinoa* leaves. The presence of the virus in lesions was confirmed by the Northern blot hybridization (data not shown). One representative of these clones, signed pPLDS-10 (Fig. 1), was randomly selected for sequencing and was found to differ from pPLDS-2 at six nt positions within ORF (p27) and/or ORF (p87). Which of these nucleotide changes reversed the non-infectivity of pPLDS-2 is currently under investigation. Despite the biological activity displayed by pPLDS-10 derived transcripts, the number of lesions that appeared on the inoculated leaves was considerably lower than that observed with the viral RNA as inoculum (Fig. 1 and data not shown). Therefore, we followed two approaches to test the effect of additional nucleotides at the 5' and/or 3' terminus of the transcripts on their infectivity. The viral full-length cDNA inserted into pPLDS-10 was amplified by PCR and ligated into pUC18-derived plasmid. This plasmid contained the CaMV 35S promoter and the terminator sequence from the nopaline synthase gene in such a way that RNA polymerase II would initiate transcription exactly at the first nucleotide of the viral sequence. *C. quinoa* leaves inoculated with the resulting construct (approximately 2 µg

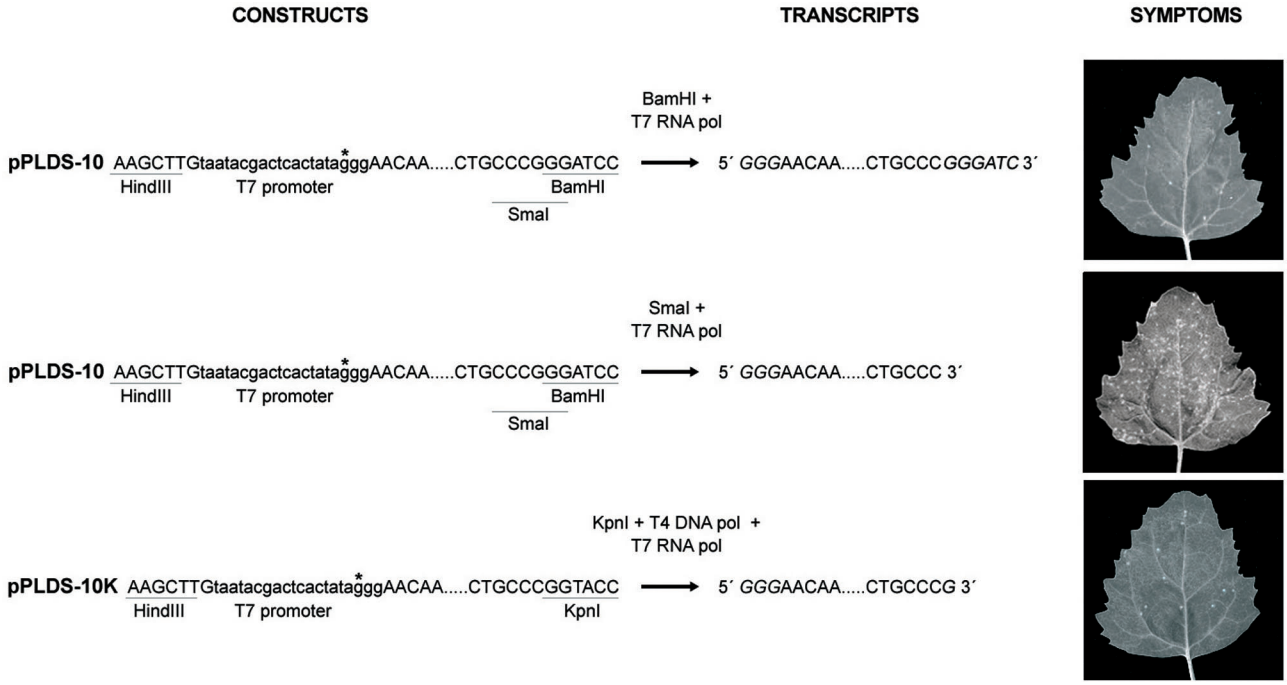

Fig. 1

Schematic representation of the generation of transcripts from constructs pPLDS-10 and pPLDS-10K

The nucleotide sequences and restriction sites are shown at the borders of the full-length viral cDNA. The initiation site for transcription is indicated by asterisk and non-viral nucleotides in the transcripts are shown in italics. Photographs at the right show the lesions induced by the different transcripts on *C. quinoa* leaves.

per leaf) developed lesions similar in number to those induced by transcripts synthesized in vitro from *Bam*HI digested-pPLDS-10, although their appearance was delayed 1–2 days with respect to the transcript-inoculated plants.

In view of the above result, the possible effect of the extra nt present at the 3' terminus on the infectivity of the transcripts was investigated. The viral cDNA inserted in pPLDS-10 was amplified by PCR with primers CH61 and CH89 (5'-ACGTGGTACCGGGCAGATCAGGGGGGT GGGTTAC-3'), complementary to the 3' terminus of the viral sequence (nt 3859–3884) with a *Kpn*I site (underlined) at the 5' terminus. The PCR product was digested using *Hind*III and *Kpn*I and ligated to the properly digested pUC18. The resulting recombinant plasmid signed as pPLDS-10K was used as a template to synthesize transcripts after its linearization with *Kpn*I and subsequent polishment with T4 DNA polymerase (Roche). In addition, new transcripts were synthesized from the construct pPLDS-10 linearized with *Sma*I. The number of lesions induced by the transcripts derived from the *Sma*I digested plasmid that were expected to contain a deletion at the 3' terminus was about 10-fold higher than number of lesions induced by the transcripts from the *Bam*HI linearized construct or by the *Kpn*I plus T4 DNA polymerase treated pPLDS-10K template that were predicted to contain the precise 3' end (Fig. 1). This result strongly supported the idea that the G nt previously determined as the 3' terminal residue of the PLPV genome (Castaño and Hernández, 2005) is not in fact present in the viral ssRNA. The replicating form of double-stranded RNA (dsRNA) in several positive RNA viruses carries an extra residue at the 3' terminus of the plus and/or minus strand (Karasev *et al.*, 1995; Galiakparov *et al.*, 1999). Since this kind of dsRNA was the template used for cloning and sequencing of the PLPV 3'-proximal region, our present observations indicated that PLPV as well as the remaining members of the family *Tombusviridae* ended with CCC-OH (White and Nagy, 2004).

In addition, the transcripts derived from the *Sma*I digested pPLDS-10 construct were used to inoculate *N. clevelandii* and *N. benthamiana* plants. The inoculated plants did not develop obvious symptoms, but the viral RNA was detected by Northern blot hybridization in both local and systemic leaves like in the control plants inoculated with the parental virus (Fig. 2).

In conclusion, we have generated a full-length cDNA from PLPV and obtained the infectious transcripts. The

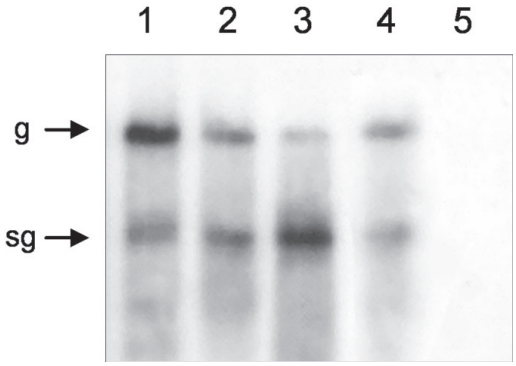

Fig. 2

Northern-blot hybridization of total RNA from plants inoculated with transcripts derived from *Sma*I digested pPLDS-10

RNA samples were prepared from inoculated leaves of *C. quinoa* (2) or from systemic leaves of *N. clevelandii* (3) and *N. benthamiana* (4). RNA preparations from *C. quinoa* plants infected with PLPV isolate PV-0193 (1) and from the mock-infected plants (5) were included as positive and negative controls, respectively. The arrows indicate PLPV genomic (g) and subgenomic (sg) RNA.

results have shown a strong effect of additional nucleotides at the 3' terminus of the viral RNA on infectivity of transcripts, whereas three extra residues at the 5' terminus did not substantially interfere with the viral multiplication. Our results were in contrast with those reported for other members of the family *Tombusviridae* as *Pothos latent virus* or *Melon necrotic spot virus*, which infectivity was not compromised by 3' extensions in the viral RNA (Rubino and Russo, 1997; Díaz *et al*., 2003). To deepen our knowledge of PLPV genomic organization and to clarify the involvement of each viral protein in PLPV basic biology, at this time reverse genetics experiments are being performed using the biologically active cDNA clone.

Acknowledgements. This study was supported by the grants AGL2000-0942 and AGL2003-04249 from the Ministerio de Ciencia y Tecnología de España and by the grants Grupos 03/064 and ACOM06/210 from the Generalitat Valenciana. A.C. was the recipient of predoctoral fellowships from the Generalitat Valenciana and from CSIC-Fundación Bancaja.

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