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QUARTERLY

Infectious transcripts from cloned cDNA of potato leafroll luteovirus*

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Infectious transcripts play a key role in the research on plant viruses at the molecular level. A number of cDNA clones covering the whole genome of the Polish isolate of potato leafroll virus were constructed. Four overlapping clones were selected and assembled using restriction sites. The full copy was positioned between T7 RNA polymerase promoter and unique *Sca*I site. The full-length capped transcripts of the sequence of the viral genome synthesised *in vitro* were able to replicate in protoplasts and to produce the viral coat protein.

The strategy of using infectious RNA transcripts from cDNA clones to investigate the molecular biology of RNA viruses has been used successfully with several plant viruses [1, 2]. The full-length cDNA copy of the viral genome fused to bacteriophage RNA promoter active *in vitro* is used as a template for the synthesis of RNA molecules identical to, or closely resembling the RNA of the viral genome. The synthesised transcripts can be used for the protoplast inoculation and, in some cases, for direct infection of the host plant. RNA transcripts of cDNA copies of viral genomes not only provide a source of sufficient amounts of genetic material but also enable deeper insight into viral gene expression and function using site-directed mutagenesis of the corresponding cDNA clone.

Potato leafroll virus (PLRV) is a member of the luteovirus group II of plant viruses [3].

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Abbreviations: CP, coat protein; nt, nucleotide(s); ORF, open reading frame; PLRV, potato leafroll virus; RT, readthrough domain; SSC, saline-sodium citrate; TEA, Tris/EDTA acetate; VPg, viral protein genome-linked.

Several members of this group cause important world-wide economic losses in plant crops [4]. The isometric virions of luteoviruses (23 nm) are assembled from the virion coat protein and a second polypeptide known as the readthrough protein. To a single-stranded positive-sense monopartite RNA of PLRV genome which is about 6 kb long, a small protein (VPg) is covalently linked [5]. Like the other luteoviruses, location for PLRV is generally limited to the phloem tissue and it is not mechanically transmissible but it is spread by aphids (*Myzus persicae*) in a persistent, circulative manner [6, 7]. Several complete nucleotide sequences of different geographical isolates of PLRV have been reported so far [8–11]. They all show a high degree of similarity at the nucleotide level, ranging from 93% to 97%, and have the same genome organisation. Six major open reading frames (ORFs) are arranged on the genomic RNA into 5′proximal (ORF0, ORF1 and ORF2) and 3′ proximal (ORF3, ORF4 and ORF5) gene clusters which are separated by a small intergenic region (Fig. 1A). The genomic RNA serves as a template for translation of the genes of 5′ cluster [8] whereas the 3′ located genes are translated from 2.3 kb subgenomic RNA [12, 13].

Infectious clones (full length cDNA) for several luteoviruses have been obtained [14–17].

Here we report on the construction of full-length cDNA clones of the PLRV Polish isolate (PLRV-P) fused to the bacteriophage T7 RNA polymerase promoter. *In vitro* RNA transcripts directed by T7 RNA polymerase are infectious when introduced into tobacco protoplasts.

MATERIALS AND METHODS

Viruses. All experiments were performed with the Polish isolate of PLRV (PLRV-P) from the collection of the Institute for Potato Research in Młochów. Virions were purified using sucrose gradient [18] from the systemically infected potato plants (cv. Osa). RNA was extracted by the guanidinium thiocyanate method [18] and its quality checked by agarose/TAE gel electrophoresis.

The nucleotide sequence of this isolate was recently published [11].

cDNA synthesis and cloning. The construction strategy of the full-length cDNA clone of PLRV is given in Fig. 1. The first cDNA strand was synthesised on the template of purified RNA by Moloney murine leukaemia virus reverse transcriptase as described previously [11]. The second strand of cDNA was synthesised either by DNA polymerase I or by Taq polymerase in PCR or *in vivo*.

For cDNA cloning, analysis and construction of the full-length copy of PLRV genome widely known procedures were used [19].

Four overlapping clones pBC, pES, pVAP and pTB were selected for the full-copy assembly of pJF (Fig.1b). pBC (nt 1–665 of the viral genome) was obtained in PCR using B1 primer: 5′ G C T T C T A T G C T G A A A G T C C A G complementary to the region 645–665 nt of the viral genome and H1 primer: 5′ *T A A T A C G A C T C A C T A T A* G A C A A A A G A A T A C C A G G G G A A A containing T7 promoter (in italics) and viral 5′ end sequence (underlined). pES (nt 590–3930) was obtained with the P3 primer: 5′GAAGTAAGATGCTTGTGATC complementary to the region 3920–3939nt of the viral genome. The second strand was synthesised by DNA polymerase I from *E. coli*. Clone pVAP (nt 3126–5466) was obtained previously [11]. pTB (nt 4444–5882) was obtained in PCR using H10 primer: 5′ C T T A G G G A G C C A A A G G A T G A of the sequence of nt 4361–4380 of the viral genome and H2 primer: 5′ *A G T A C T* A C T C A A C C C T G T A A G A G G containing the sequence of *ScaI* restriction site (in italics) and a sequence complementary to the nt 5862–5882 of the viral genome (underlined).

The full-length clone was constructed gradually. In the first step two clones, pBM and pVB, were obtained, containing 5′ and 3′ re-

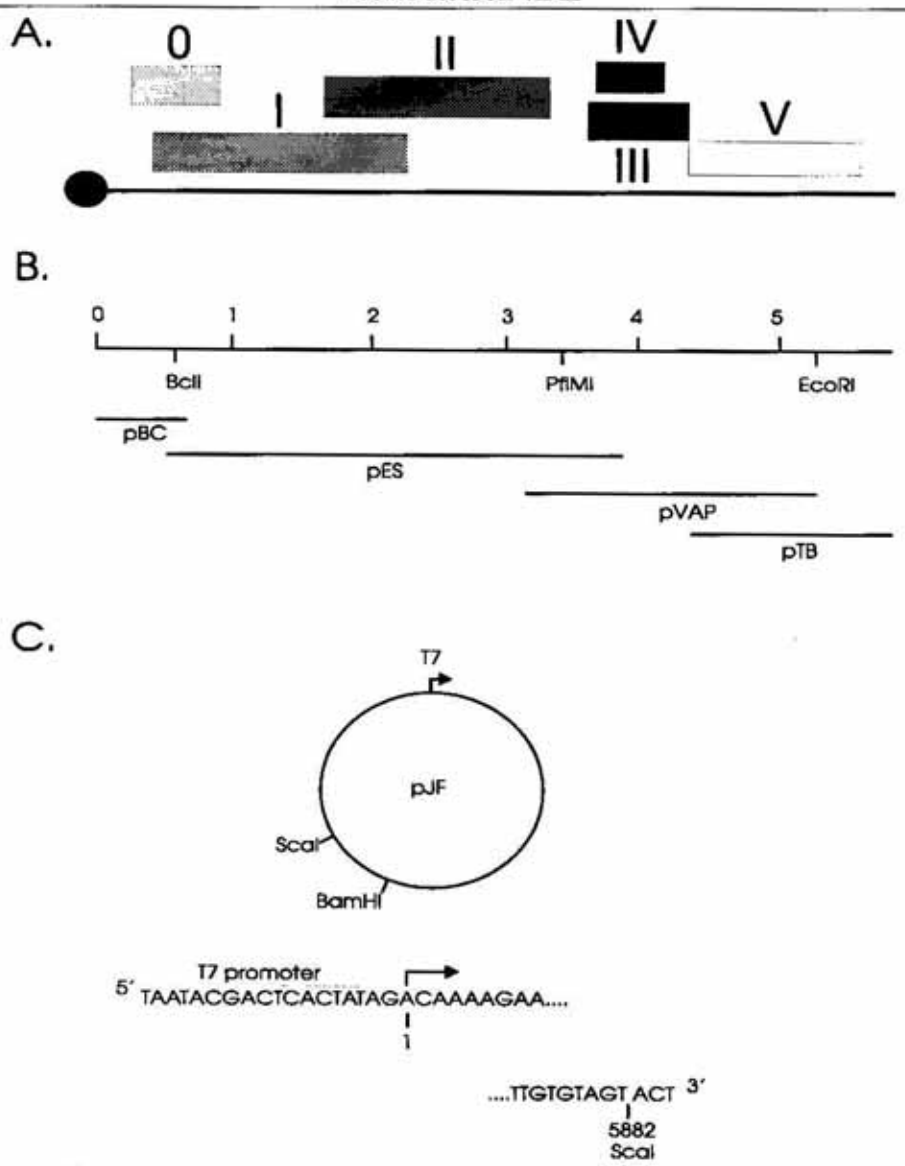

Figure 1. Construction of the full-length cDNA copy of the PLRV genome.

A. The organisation of the PLRV genome. B. cDNA clones selected for assembly of the full copy; restriction sites used for cloning are marked. C. The restriction map of pJF and the sequence of the 5′ and 3′ extremities of the full copy of the viral genome.

gions of PLRV genome, respectively. The clone pBM (nt 1–3930) contained viral cDNA of pBC and pES connected at *Bcl*I restriction site (position 630 nt), whereas the clone pVB (nt 3126–5882) carried cDNA of pVAP and pTB after *Eco*RI digestion and ligation (position 5176 nt). Finally, cDNA fragments located on pBM and pVB were connected at *Pfl*MI site (position 3350 nt) and cloned in pJRD184 [20]. The resulting clone pJF contains the full-length cDNA copy of PLRV under the control of T7 RNA polymerase promoter designed in the PCR primer.

In vitro *transcription and translation.* *In vitro* transcription was performed by bacteriophage T7 RNA polymerase (BRL) in a buffer supplied by manufacturer. Transcripts after purification were analysed by agarose/TEA electrophoresis and visualized by ethidium bromide staining. DNA was removed from the transcript preparations by RQ1 DNase RNase free (Promega) according to the manufacturer's instructions.

In vitro translation of 500 ng of purified transcripts and viral RNA was performed in wheat germ translation kit supplemented with

[^{35}S]methionine and tRNA according to manufacturer's instructions (Boehringer Mannheim). Proteins were separated by SDS/PAGE [21] and analysed by autoradiography.

Infection of tobacco protoplasts and analysis of inoculated protoplasts. Protoplasts were isolated from mature non-flowering tobacco plants cv. Xanthi [22]. Approximately 10^6 protoplasts in 1 ml of electroporation buffer were used for electroporation with 1 μg of the viral genomic RNA or 5 μg of one of the transcripts. Immediately after electroporation the protoplasts were suspended in 10 ml of a culture medium and incubated at 25°C in the dark for 3 days.

Approximately 100000 protoplasts were harvested by centrifugation at 100 × *g*, 5 min. Total cellular RNA was isolated as described by Weiland & Dreher [23]. After fractionation in formaldehyde-agarose gel [23] RNA was blotted to a nylon membrane by capillary transfer in 10×SSC. Viral RNA was detected by non-radioactive Northern hybridisation with two antisense RNA probes (complementary to the residues 174–1841 and 3126–5176) labelled with digoxygenin-UTP (Boehringer Mannheim) according to manufacturer's instructions.

For the analysis of proteins, protoplasts were harvested as above. Total protoplast proteins were separated by SDS/PAGE [21], transferred onto a nitrocellulose membrane and analysed by western blotting [24] with commercially available anti-PLRV antibody conjugated with alkaline phosphatase (Boehringer Mannheim).

RESULTS

Cloning, transcription and translation of the full-length PLRV-P cDNA

The full-length cDNA clone of PLRV genome, pJF (1–5882nt) constructed from existing clones as described in Methods was cloned under control of the bacteriophage T7 RNA polymerase promoter. The strategy of its construction is outlined in Fig. 1b. In the construct pJF only one non-viral residue (G) was left at the 5′ terminus. It has been established with other infectious clones that long 5′ non-viral extensions can interfere with transcripts infectivity [1, 25].

Single-stranded RNA of the sequence of the viral genome was obtained by *in vitro* transcription of the *Sca*I-linearized plasmid pJF by T7 RNA polymerase. Transcription was performed either in the presence or absence of the cap analogue m7(5′)Gppp(5′)G. Truncated transcripts synthesised on the template of plasmids linearized with *Bam*HI (position 4167 nt) served as the negative control (Fig. 1c).

Full-length transcripts obtained showed the same mobility on electrophoresis as PLRV RNA. A comparison of the amount of the transcript and DNA template in the gel indicated that the efficiency of the transcription was about 5 RNA molecules per one template.

In vitro translation of viral RNA leads to the synthesis of two main protein products of molecular mass of 28 kDa and 70 kDa, corresponding to ORF0 and ORF1 products, respectively [8]. In order to check whether the transcripts obtained *in vitro*, used as templates in translation system *in vitro* give the same proteins, the transcripts were translated *in vitro* by wheat germ lysate in the presence of [^{35}S]methionine. The results presented in Fig. 2 indicate that proteins synthesised on the template of *in vitro* transcripts, and viral RNA are the same.

Biological activity of *in vitro* transcripts

Northern blot analysis of protoplasts electroporated with the PLRV RNA (positive control) and capped full-length RNA transcripts showed the presence of the genomic and subgenomic viral RNAs (Fig. 3, lane 2 and 5, respectively). They both were absent, however, from the protoplasts inoculated with the un-

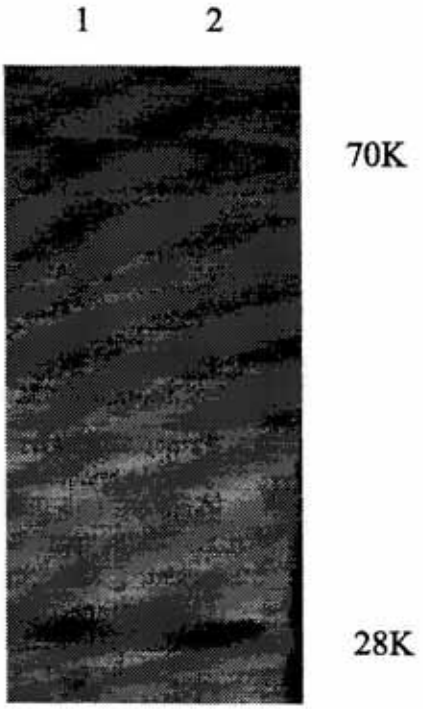

Figure 2. Autoradiogram of *in vitro* translation products.

Proteins obtained from translation of pJF *in vitro* transcript (lane 1) and of viral RNA (lane 2). Molecular mass of proteins indicated on the right.

capped full-length transcripts (lane 4). Viral RNA was also lacking in the case of protoplasts inoculated with the truncated transcript and the mock-inoculated protoplasts serving as the negative control (lane 3 and 1, respectively).

In the Western blot analysis of proteins of protoplasts inoculated with PLRV RNA or the full-length capped transcript two bands of 23 kDa (corresponding to the coat protein) and 80 kDa (corresponding to the fusion of the coat protein and the readthrough domain) were visible (Fig. 4, lane 5 and 6, respectively). In the preparation of purified virions two bands of 23 kDa (coat protein) and 55 kDa (truncated fusion protein) were detected (lane 2). No signal was present in the proteins of protoplasts inoculated with the truncated transcript or mock-inoculated protoplasts (lane 4 and 3, respectively).

DISCUSSION

The low concentration of luteoviruses attained in infected tissue and the lack of mechanical transmissibility of either virus or viral RNA impeded the investigation of their gene expression at the molecular level. *In vitro* synthesis of biologically active RNA transcripts from full-length cDNA clones and the construction of viral cDNA genome under control of the cauliflower mosaic virus 35S promoter [26] enabled a significant progress in understanding the biological function of luteoviral proteins. Recent progress in understanding the biological function of different luteoviral proteins in virus life cycle (including replication, movement and aphid transmission) has been obtained by reverse genetics using transcripts of full-length cDNA or infectious cDNA clones into which specific mutations were introduced [27–30].

In this paper we report the construction of the transcripts of PLRV infectious *in vitro*. The strategy described in Materials and Methods enabled to construct the full-length cDNA copy of the viral genome under T7 phage promoter and precisely programmed the *in vitro* transcription start and termination points. The sequence of the full-length RNA molecules synthesised *in vitro* differed from the sequence of the viral genomic RNA only by an additional G residue at the 5′ end.

For *in vivo* experiments transcripts were synthesised either in the presence or absence of the cap analogue, m7(5')Gppp(5')G. The capped, full-length transcripts showed in protoplasts the ability to replicate and to produce the subgenomic RNA. In contrast, uncapped full-length transcripts showed no activity in protoplasts. This phenomenon points to the necessity of the cap analogue for biological activity of transcripts of PLRV. The same observation was made for BWYV [31] and it has been suggested that the cap analogue might play the role of VPg normally present at the 5′ end of the viral genomic RNA. VPg as well as the cap structure can increase RNA stability

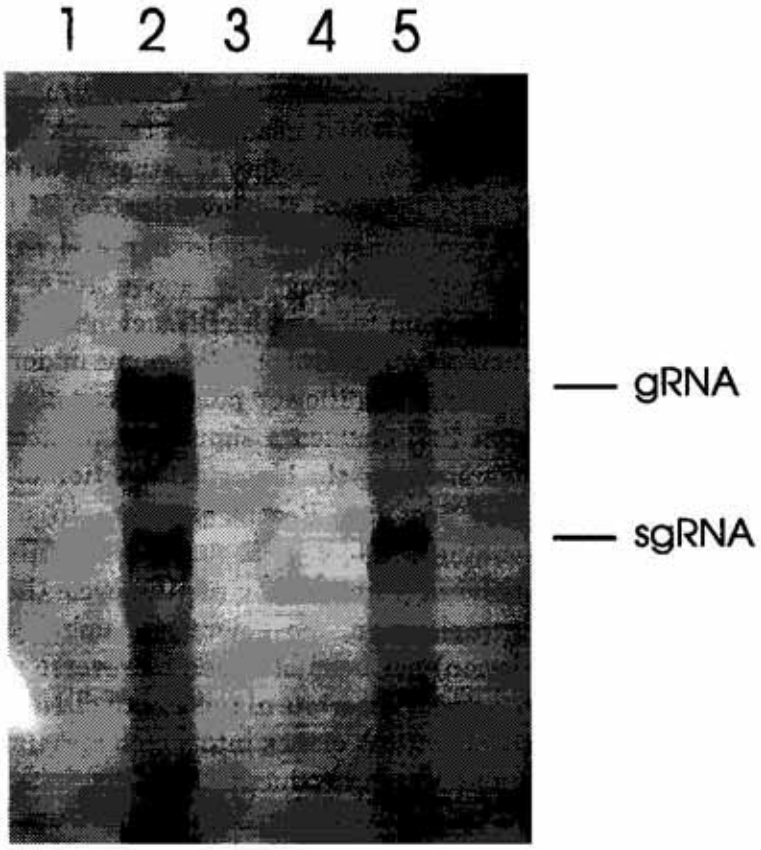

Figure 3. Northern analysis of protoplast RNA.

Protoplasts were: mock-inoculated (lane 1), inoculated with PLRV RNA (lane 2), inoculated with truncated transcript (pJF/*Bam*HI, lane 3), inoculated with the full-length uncapped transcript (pJF/*Sca*I, lane 4), inoculated with the full-length capped transcript (pJF/*Sca*I, lane 5). Positions of the genomic RNA (gRNA) and subgenomic RNA (sgRNA) are indicated.

and/or efficiency of its translation. Interestingly, in the case of infectious transcripts of BYDV, a member of the group I of luteoviruses, the supplement of cap analogue caused only a doubling of the infectivity of transcripts [32]. The truncated transcripts (pJF/*Bam*HI) used as the negative control did not replicate since the signals for initiation of (-) strand synthesis are located at the 3′ end of the genomic RNA.

As it was shown by Western blotting, the viral coat protein and the fusion coat protein-

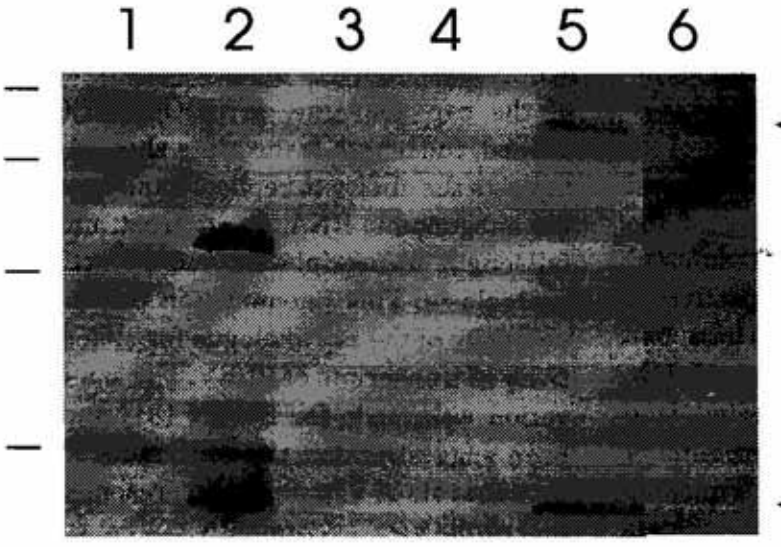

Figure 4. Western blotting analysis of protoplast proteins.

Positions of the molecular mass standards (lane 1) are indicated by lines: 18.4 kDa, 29.0 kDa, 43.0 kDa, 68.0 kDa, 97.4 kDa. As a control a PLRV virion preparation is included (lane 2). Protoplasts were mock-inoculated (lane 3), inoculated with the truncated transcript (pJF/*Bam*HI, lane 4), inoculated with the full-length capped transcript (pJF/*Sca*I, lane 5), inoculated with the PLRV RNA (lane 6). Position of the viral coat protein is indicated by the lower arrow; position of the fusion coat protein-readthrough protein is indicated by the upper arrow.

readthrough domain can be found in the protoplasts inoculated with the full-length capped transcripts. The appearance of these viral proteins in transfected protoplasts is an additional evidence of the biological activity of the pJF clone since both these structural proteins are translated from the subgenomic RNA and not from the input RNA. In our Western blots we found a difference in size between the fusion coat protein-readthrough domain from the virion preparation and from the protoplasts (Fig. 4, lane 2 *versus* lane 5 and 6). However, the truncated form of readthrough domain normally appears in the preparation of virions in contrast to the untruncated form in the cell [33]. The presence of the coat protein and ORF6 product suggests that it should be possible to complete the infection cycle by feeding the aphids on inoculated protoplasts and transferring the insects to the host plants.

The obtained infectious transcripts of PLRV create a novel system for molecular research studies on this virus. In the future additional experiments with site-directed mutagenesis will provide more information about molecular mechanisms of PLRV pathogenesis and involvement of products of particular ORFs in the life cycle of this economically important virus.

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