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BRIEF REPORT

Construction of an infectious full-length cDNA clone of potato virus M

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Abstract An infectious full-length cDNA clone of potato virus M (PVM) was produced. Total RNA was extracted from PVM-infected *Nicotiana hesperis* plants and used for cDNA synthesis. Subsequent RT-PCR produced two DNA fragments of about 5.5 and 3.2 kbp, which were ligated downstream of an enhanced 35S cauliflower mosaic virus promoter. After cloning of the enhanced 35S promoter with the PVM sequence into a modified pBIN19 plasmid and electroporation of *Agrobacterium tumefaciens*, the agroinoculated PVM full-length clone (pPVM-flc) led to systemic PVM infections in different host plants, causing symptoms indistinguishable from those caused by wild-type PVM.

Potato virus M (PVM) occurs worldwide in nearly all potato-growing areas. Depending on potato cultivar and environmental conditions, PVM infections can cause yield losses of about 25–50% [38]. The foliage of PVM-infected potato plants shows symptoms like mottle, mosaic, crinkling, rolling and leaflet deformation, although symptom expression can range from extremely mild to severe [11, 38]. PVM is a member of the genus *Carlavirus* [28, 30, 43] and was first described in 1923 [32]. Its filamentous particles are composed of multiple coat protein subunits encapsidating a monopartite plus-sense ssRNA genome of approximately 8.5 kb [43]. The polyadenylated PVM RNA possesses a cap structure at the 5′ end [42] and contains six open reading frames (ORFs). ORF1 encodes a protein that contains methyl transferase, helicase and polymerase domains typical of those found in proteins involved in RNA replication [15, 16, 29]. ORFs 2, 3 and 4 overlap and form a so called "triple gene block" (TGB). The proteins encoded by these ORFs are similar to those of potexviruses, which have been shown to interact with one another and with the coat protein and are mainly involved in cell-to-cell movement [2, 3, 13–15, 17, 23]. ORFs 5 and 6 encode the coat protein and a cysteine-rich nucleic acid binding protein, respectively [8].

In general, carlaviruses like PVM have not been as extensively characterised as plant viruses of other genera. There is only a limited number of complete genome sequences available for members of this genus, including aconitum latent virus [7], blueberry scorch virus [4], chrysanthemum virus B [26], garlic latent virus [35], hop latent virus [9, 10], ligustrum necrotic ringspot virus [33], lily symptomless virus [5], passiflora latent virus [36], PVM [43], poplar mosaic virus [34], daphne virus S [19] narcissus common latent virus [44] and sweet potato chlorotic fleck virus [1]. In addition, infectious full-length clones are available only from blueberry scorch virus [18] and poplar mosaic virus [25]. Therefore, we decided to produce an infectious full-length clone of PVM and to investigate its capability to infect different plant species.

The PVM isolate (PV0273) kindly provided by the plant virus collection of Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) was propagated and maintained in *Solanum esculentum* Mill. cv. Lizzy and *Nicotiana hesperis*. Total RNA from 0.1 g infected *N. hesperis* leaves was prepared using a silica-based extraction method [21]. The 5′ end of the PVM genome was determined by direct ATP-tailing of the 3′ end of the antisense strand of extracted viral dsRNA [24] using yeast poly(A) polymerase (USB) according to the

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manufacturer's instructions. Synthesis of cDNA and amplification of the tailed antisense strand were performed as described for the 3′ end (3.2-kbp fragment) of the genomic viral RNA.

For construction of an infectious PVM full-length cDNA clone, the known PVM sequence NC_001361 (GenBank, NCBI) and the sequence of a previously constructed but non-infectious PVM clone were aligned, and this revealed a single *Afl*II restriction site in both sequences. Accordingly, oligonucleotides were designed with *Afl*II sites or with additional restriction sites at their 5′ ends that were not present in the PVM genome. This approach aimed at amplifying the whole viral genome after reverse transcription by PCR in two DNA fragments and utilizing the introduced restriction sites for cloning. Briefly, two independent cDNA templates were generated from 1 µl of purified total RNA in a two-step procedure, using Expand Reverse Transcriptase (Roche) and the antisense primers PVM3as (AATCTAGATTTTTTTTTTTTTTTTTTTTTTGGCTAAAAATAGTTAAAAACCAA) or PVMSFas (GCTCTAGAGCCTGGCAGCCTTAAGTACTTCATCAATTCCAG), according to the manufacturer's recommendations, in 20-µl reaction volumes. After cDNA synthesis, two DNA fragments of about 5.5 and 3.2 kbp were obtained using 0.4 µl of cDNA and Phusion™ High-Fidelity DNA Polymerase (Finnzymes, 0.4 U), according to the manufacturer's protocols, in 20-µl reaction volumes. For amplification of the 3.2-kbp fragment, which represents the 3′ third of the viral genome, the primer pair PVMSF2s (AGTACTTAAGGTTACCAAGTGATCTAATCGAGGATTACA) and PVM3as was used with the following PCR conditions: 1 cycle of 98°C for 30 s, followed by 35 cycles of 98°C for 10 s, 60°C for 20 s and 72°C for 180 s and a final extension step of 72°C for 10 min. The primer pair PVM1s (ATGGCGCGCCGGATAAACAAACATACAATA) and PVMSFas was used for the amplification of the 5.5-kbp DNA fragment, representing the 5′ two-thirds of the PVM genome, with an annealing temperature of 65°C and an extension time of 315 s.

For creating the PVM full-length clone (pPVM-flc), the larger PCR fragment was digested using *Asc*I and *Xba*I followed by ligation into a modified plasmid directly downstream of the enhanced cauliflower mosaic virus (CaMV) 35S promoter [40]. The plasmid was propagated in *Escherichia coli* NM522, and plasmid DNA was isolated from overnight cultures using standard protocols. For the second cloning step, five clones containing the 5′-terminal PVM fragment were mixed. The 3.2-kbp PCR fragment was excised and extracted from the agarose gel (Gel Extraction Kit, Qiagen). It was then A-tailed at 70°C for 1 h by using a heat-stable DNA polymerase purified according to 27 [27] in a 10-µl reaction volume consisting of 7.3 µl PCR fragment, 1 µl 10× reaction buffer (0.1 M Tris–HCl (pH 8.8), 0.5 M KCl and 0.8% (V/V) NON-IDET® P40), 1 µl $MgCl_2$ (25 mM) and 0.2 µl of 10 mM dATP. The tailed fragment was cloned into a T-vector, and six recombinant clones were mixed before integrating the 3.2-kbp fragment directly downstream of the 5.5-kbp fragment (Fig. 1) by using the *Afl*II and *Xba*I restriction sites, localised at the 5′-ends of the primers (PVMSF2s and PVM3as). Finally, from a mixture of 21 recombinant plasmids, containing the enhanced CaMV 35S promoter and the PVM genome, the entire cassette was purified (Gel Extraction Kit, Qiagen) and subcloned into a modified pBIN19 plasmid using *Cla*I and *Xba*I, resulting in pPVM-flc, which was electroporated into the *Agrobacterium tumefaciens* strain LBA4404 [12].

The sequence of the constructed pPVM-flc reported here was determined by a gene walking approach. For this, 24 primers, located about 350 nucleotides apart, were designed in order to obtain overlapping sequences. The complete PVM (DSMZ, PV0273) sequence was 8,523 nucleotides, excluding the 3′-poly(A) tail. Analysis of the contig sequence revealed a typical PVM genome organisation. Comparative sequence analyses using ClustalX [39] showed the highest overall nucleotide sequence identity (95%) with a Russian isolate (accession no. NC_001361). The deduced amino acid sequences of the individual ORFs had identities ranging from 90 to 98% with those of other PVM isolates.

In the first set of experiments, recombinant *A. tumefaciens* LBA4404 cells were incubated in inoculation buffer (10 mM $MgSO_4$, 10 mM MES and 100 µM acetosyringone), and the suspension was injected into the abaxial intercellular space of three to four leaves of three different individual plant species (*Nicotiana benthamiana*, *N. hesperis* and *S. esculentum* Mill. cv. Lizzy) [6]. Systemic PVM infections were serologically detected in all of

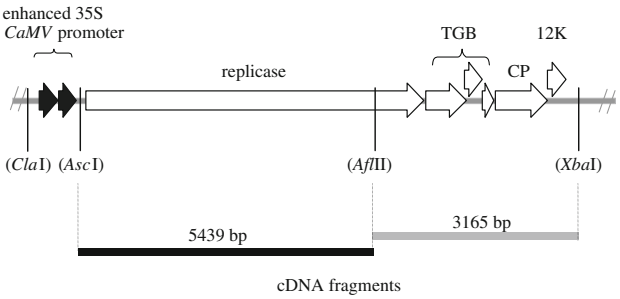

Fig. 1 Schematic representation of the pPVM-flc structure. PVM ORFs (*replicase* gene for replication-associated protein, *TGB* triple gene block, *CP* coat protein, *12 K* 12 K nucleic acid binding protein) are indicated as *white arrows*. The enhanced 35S CaMV promoter is represented by *black arrows*. The whole PVM genome was amplified in two fragments (*black* and *grey rectangles*). The positions of the different restriction endonuclease sites used for construction of the PVM full-length clone are indicated

Table 1 Type and number of systemically infected plant species and cultivars after agroinoculation with PVM-flc and mechanical inoculation with PVM-wt

Plant species	Mechanical inoculation (dpi)	Agro-inoculation (dpi)
Solanaceae		
Capsicum annuum L. cv. California Wonder	0/6[a] (39)[b]	0/6 (39)
Nicotiana rustica L.	0/12 (38)	0/12 (37)
Nicotiana hesperis	3/3 (48)	1/3 (48)
Nicotiana tabacum L. cv. Xanthi-nc	0/12 (38)	0/12 (37)
Nicotiana Samsun "nn"	0/12 (38)	0/12 (37)
Nicotiana occidentalis	12/12 (28)	11/12 (28)
Nicotiana glutinosa	8/12 (30)	9/12 (30)
Nicotiana benthamiana	6/6 (35)	3/6 (35)
Nicotiana edwardsonii	0/6 (30)	0/6 (30)
Lycopersicon esculentum Mill cv. ACE55	6/6 (29)	3/6 (28)
Lycopersicon esculentum Mill cv. Harzfeuer	6/6 (29)	2/6 (28)
Lycopersicon esculentum Mill. cv. Luxor	6/6 (29)	4/6 (28)
Solanum nigrum L.	6/6 (52)	5/6 (47)
Fabaceae		
Pisum sativum L. cv. Alaska	0/6 (17)	0/6 (17)
Pisum sativum L. cv. Senator	0/6 (17)	0/6 (17)
Chenopodiaceae		
Chenopodium capitatum (L.) Asch.	0/3 (26)	0/3 (26)
Caryophyllaceae		
Dianthus barbatus L.	0/6 (32)	0/6 (32)

[a] Infected/inoculated plants

[b] Time of testing after inoculation (dpi)

the agroinoculated plants 4 weeks after agroinfiltration, using alkaline phosphatase-labelled antibodies to PVM (Loewe Biochemica) in a tissue blot immunosorbent assay (TBIA) [20]. After fast red/naphthol chromogenic staining, purple-coloured foci on the membranes indicated the presence of PVM antigen. PVM coat protein was also detected in agroinoculated *N. hesperis* plants by western blot immunoassay. For this purpose, total protein extracts [2] were subjected to tricine-SDS-polyacrylamide gel electrophoresis [31]. Then, proteins were electroblotted onto a nitrocellulose membrane [41], and the viral coat protein of about 34-kDa was detected using the aforementioned antibodies. In addition, PVM particles were detected in agroinoculated *N. hesperis* plants by electron microscopy after negative staining with 2% uranyl acetate [22]. No differences in particle morphology were observed between leaf extracts from pPVM-flc and wild-type PVM (PVM-wt)-infected plants. Moreover, symptoms of pPVM-flc-infected plants were indistinguishable from those infected with PVM-wt. In contrast to systemically infected tomato and *N. benthamiana* plants, all of which remained symptomless, infected *N. hesperis* plants showed conspicuous mosaic symptoms on the leaves. Additionally, leaves were reduced in size, had a tubular shape and exhibited curling, crinkling and leaf distortions.

In another experiment, pPVM-flc was transferred into *A. tumefaciens* strain C58C1 by electroporation. This was done because aggregation of LBA4404 cells had caused problems in previous agroinoculation experiments with beet mild yellowing virus, whereas C58C1 cells grow more uniformly and are less prone to aggregate [37]. To investigate differences in host range of PVM originating from the full-length clone, different plant species were agroinoculated either with pPVM-flc or mechanically inoculated with plant sap of PVM-wt-infected *N. hesperis*. All plants were tested for PVM infection by TBIA from 17 to 52 dpi. The results are summarised in Table 1.

Systemic infection with PVM was achieved by mechanical inoculation of PVM-wt as well as by agroinoculation with pPVM-flc in 8 out of the 17 plant species tested. However, no differences in host range and symptom development were observed between agroinoculated (pPVM-flc) and mechanically inoculated (PVM-wt) plants. Plant species insusceptible to mechanical inoculation with PVM were also not infected by agroinoculation with PVM-flc.

This work represents the first infectious full-length cDNA clone of a PVM isolate. Previously, the successful synthesis of infectious blueberry scorch virus transcripts [18] and the generation of a full-length clone of poplar mosaic virus [25] have been published for the genus *Carlavirus*. The generated pPVM-flc was shown to cause systemic infections in different plant species and might be useful in future experiments, e.g. for analysing gene

functions, studying recombination of carlaviruses and screening for PVM resistance in potato breeding programs.

The determined sequence has been deposited in GenBank under accession no. EU604672.

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