**Short communication**

**Cell-free cloning and biolistic inoculation of an infectious cDNA of potato virus Y**

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Potato virus Y (PVY) full-length cDNA has been found to be refractory to cloning in *Escherichia coli* cells. A full-length 9.7 kb PVY cDNA was obtained by reverse transcription polymerase chain reaction (RT-PCR) from the RNA of PVY (tuber necrotic strain, PVYNNTN). Double-stranded DNA fragments were used as primers (ds megaprimers), to include signals for transcription in vivo (a cauliflower mosaic virus 35S RNA promoter and a nopaline synthase terminator) in the final PCR product. Biolistic bombardment with a helium particle gun was used to inoculate the amplified product to detached tobacco leaves. Inoculation of tobacco plants with ground inoculated leaves followed by northern blot, ELISA and immuno-electron microscopy demonstrated that the DNA was highly infectious with up to 90% of bombarded leaves containing the virus. This methodology will allow the use of reverse genetics in the study of PVY–plant interactions and will also be useful for obtaining infectious cDNA from other viruses with large RNA genomes.

Potato virus Y (PVY) is the type member of the potyvirus group, the largest group of plant viruses. It infects solanaceous crops (potato, tomato, tobacco, pepper) worldwide. Potyvirus particles are flexuous rods about 680–900 nm in length (Hollings & Brunt, 1981) and encapsidate the positive-sense, single-stranded polyadenylated RNA genome of about 10 kilobases, which is covalently linked at the 5' end to the virus-encoded VPg protein (Murphy et al., 1990). The complete nucleotide sequences of two potato virus Y strains have been established (PVYN, Robaglia et al., 1989; PVYNTN Hungary, Thole et al., 1993).

PVY is a well-known virus with many characterized strains varying in host range, symptom expression and interaction with host resistance genes (De Bokx & Huttinga, 1981; Gooding & Tolin, 1973; Gebré-Séllassié et al., 1985; Le Romancer et al., 1994). It is therefore an interesting model for studying host/pathogen interactions at the molecular level. Infectious cDNA clones from RNA virus genomes have greatly facilitated understanding of the biology of RNA viruses (for a review see Boyer & Haenni, 1994) by allowing the use of a reverse genetic approach. Infectious cDNA clones have been obtained for several potyviruses such as tobacco vein mottling virus (TVMV; Domier et al., 1989), plum pox virus (PPV; Riechmann et al., 1990; Maiss et al., 1992), tobacco etch virus (TEV; Dolja et al., 1992) and zucchini yellow mosaic virus (ZYMV; Gal-On et al., 1991).

Unfortunately, despite numerous efforts in this and other laboratories (G. Jakob & P. Malnoé, personal communication), the complete cDNA of PVY has been found refractory to conventional cloning in *Escherichia coli*. The reason for this behaviour is still obscure, but may be related to the expression of toxic products from cryptic promoters in the viral genome, since partial clones can be obtained, but all attempts to splice them together in an infectious form were unsuccessful. The recent development of polymerase chain reaction (PCR) methods for amplification of up to 35 kbp of DNA (Cheng et al., 1994; Barnes, 1994) makes it possible to generate a full-length PVY cDNA by reverse transcription–PCR (RT–PCR).

In this paper, we report a new one-step and cell-free method to generate a complete infectious cDNA from PVYNTN Liban genomic RNA using RT–PCR. This cDNA contained a built-in cauliflower mosaic virus (CaMV) 35S RNA promoter at the 5' end and the nopaline synthase polyadenylation signal at the 3' end. The PCR product was introduced directly into plant cells (*Nicotiana tabacum* var. Xanthi) using particle bombardment and led to efficient PVY infection. Particle bombardment is an effective way to introduce DNA directly into the plant cell nucleus, allowing transient or stable

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expression of genes or infection by DNA viruses (Garzon-Tiznado et al., 1990; Hagen et al., 1994). This is the first report of successful infection by a cDNA derived from an RNA virus using particle bombardment.

Virus particles were purified as described in Robaglia et al. (1989). Viral RNA was purified by phenol/chloroform extraction and ethanol precipitation. For the synthesis of full-length, first-strand PVY cDNA, 5 μg viral RNA was mixed with 100 ng of primer 1 (5’ GGCTTAATTAATTTTTTTTTTTTTTTTTTTTTTTTTTTCTCCTGATTGAAGTTTACAGTC 3’) in water, heated for 2 min at 70 °C, then incubated 1 h at 42 °C with dNTPs (1 mM each final concentration), RNase inhibitor (20 U; Boehringer) and 40 U avian myeloblastosis virus RT (Promega) in a 20 μl reaction containing the buffer supplied with the enzyme.

In order to place the viral cDNA under the control of the CaMV 35S promoter, a partial cDNA clone corresponding to the first 665 bases of PVYNN was engineered in such a way that the transcriptional start of the 35S promoter (Odell et al., 1985) corresponded to the first base of the PVY genomic RNA (Fig. 1a). A cDNA fragment corresponding to the first 665 nucleotides (nt) (up to the first HincII site; Thole et al., 1993) of PVYNN Liban was cloned in a pBluescript vector (Stratagene) after RT–PCR amplification, regenerating the HincII site at base 665. A DNA fragment containing a modified CaMV 35S promoter (from plasmid pCA2; Kay et al., 1987) was cloned upstream of the 5’ end of the viral genome. The bases between the transcription start of the 35S promoter and the first base of the PVY genome were removed by site-directed mutagenesis. A PstI–HincII DNA restriction fragment called 35S–PVY665 containing the 35S promoter linked to the first 665 bases of PVYNN cDNA was gel-purified (GeneClean; Bio 101) for use as a ds megaprimer (Barnes, 1994).

The length of the poly(A) tail was found to be an important parameter in the infectivity of transcripts of animal picornaviruses (Sarnow, 1989). To generate a primer including a long poly(A) tail, the plasmid M4/3A100 containing 4.9 kbp of the 3’ region of the PVYNN (Swiss isolate strain NN) including a poly(A) tail of about 100 residues (a gift of Gabor Jakob, Station de Recherches Agronomiques de Changins, Suisse) was digested with XhoI, which cut downstream of the inserted PVY cDNA, filled with dTTP and dCTP using Klenow
Potato virus Y genome amplification

DNA polymerase and ligated to plasmid pCAMVNOS (Fromm et al., 1986) digested by BamHI and filled with dGTP and dATP. The ligation reaction was then amplified by PCR using oligonucleotides: 5′ TAAACTTCA-ATCAGGAGCAAAAAAAAAAA 3′ and 5′ TGTCGACCCGGCTTCTAGAGATCTAGAAACA 3′ complementary to the end of the nopaline synthase terminator. The PCR product (NOS–100A–PVY19) (Fig. 1b), containing the last 19 bases of the PVY cDNA followed by the poly(A)100 tail and the nopaline synthase polyadenylation signal, was gel purified before being used as another ds megaprimer.

Megaprimers generated by PCR were blunt-ended by Klenow treatment in order to eliminate the 3′ adenylate residue added by the Taq DNA polymerase.

Of the first-strand cDNA reaction, 1 μl was diluted one hundred times with water and used in a 50 μl hot-start PCR reaction (XL-PCR; Perkin-Elmer) containing 0.2 mM-dNTPs, PCR buffer (25 mM-tricine pH 8.7, 85 mM-potassium acetate, 8% glycerol, 2% DMSO and 1.1 mM-magnesium acetate), 2 U of rTth DNA polymerase (Perkin Elmer) and approximately 1 μg of each ds megaprimer. rTth DNA polymerase is an enzyme formulation optimized in both polymerase and proof-reading activities.

PCR cycles were: 1 min at 94 °C, followed by 16 cycles of 15 s at 94 °C and 10 min at 65 °C and by 12 cycles of 15 s at 94 °C and 10 min at 65 °C with a 15 s addition at each cycle, i.e. up to 13 min. The reaction was terminated by a 10 min elongation at 72 °C. An aliquot of the final product was analysed by agarose gel electrophoresis and found to contain an approximately 10 kbp PCR product (Fig. 1c). The low molecular mass band observed is of unknown origin as it does not migrate as expected for the primers or for primer dimers.

Particle bombardment was done in the helium flow apparatus described by Godon et al. (1993), according to the protocol described. The products from one PCR reaction were purified by phenol/chloroform extraction and ethanol precipitation and resuspended at a concentration of approximately 1 μg/μl in water. One μl of DNA, which is enough for four shots, was mixed with five successive 1 μl aliquots of 95% ethanol, with vortexing between each addition, then with 11 μl of sonicated tungsten particles (100 μg/μl) kept in suspension in 95% ethanol. Of this mixture, 4 μl was immediately placed in the centre of an aluminium foil rupture disc and allowed to dry down. The disc was placed in the apparatus and helium (650 kPa) was released for 25 ms in the bombardment chamber kept under vacuum (20 kPa). Preliminary experiments showed that direct bombardment of small tobacco plants placed in the vacuum chamber was difficult; the distance between the gun and the leaf cannot be accurately...
controlled and without support the leaves tend to disintegrate in the gas flow. Young tobacco leaves were therefore detached and maintained on a 2% agar/water surface in open Petri dishes. For each shot, a leaf from a 3-4-week-old tobacco plant was used. After bombardment, the cover was replaced and the dish was placed in a plant growth chamber for 3-10 days to allow the infection to develop.

In one experiment, eight leaves were bombarded, each with approximately 0.2 µg of PCR product and after 5 days incubation in a growth chamber, each leaf was ground with 0.5 ml of inoculation buffer and used to inoculate four young tobacco plants with carborundum. Ten days later, 15 of 32 plants showed typical symptoms of PVY\textsuperscript{RMS} infection: stunting, vein necrosis and systemic necrotic spots (Fig. 2b). This experiment showed that among the 8 leaves originally submitted to bombardment, 6 were infected (Table 1). In a second experiment, 10 leaves were bombarded, each with approximately 0.5 µg of PCR product, after which they were kept for 10 days in the growth chamber. At this stage, some leaves showed faint symptoms of virus development such as vein chlorosis and interveinal curling. Each leaf was used to inoculate four young tobacco plants. In this experiment, a total of 30 out of 40 plants developed viral symptoms, showing that 9 out of the 10 initially bombarded leaves contained virus.

Altogether these results suggest that infectivity increases slightly with an increased amount of DNA (9/10 compared to 6/8) and that the extent of incubation in the growth chamber appears to significantly increase the amount of virus present in the bombarded leaves (30/40 compared to 15/32).

The presence of PVY in the retroinoculated plants was further confirmed by double antibody sandwich (DAS) - ELISA and immuno-electron microscopy (IEM; data not shown) using a commercially available antibody against PVY (Sanofi-Diagnostic) and by northern blotting of total RNA extracted from infected and control plants 10 days after inoculation according to Robaglia et al. (1993), using a PVY coat protein probe (Fig. 2a).

Note that the smear below the genomic RNA (+)strand band corresponds to (−)strand RNA molecules since a double-stranded cDNA probe has been used.

The findings reported in this paper demonstrate that it is possible to obtain an infectious PVY cDNA in a few simple steps without cloning in E. coli.

This uncloned cDNA was found to be highly infectious when applied to tobacco plants. The \textit{rTth} DNA polymerase allows good fidelity in the synthesis of the PCR product. Mistakes can still be introduced into cDNA molecules, but in the absence of a cloning step these mistakes are not propagated and a pool of molecules among which mutated ones are a minority is used. Moreover, it is unlikely to introduce the same mutation in molecules obtained in several independent syntheses.

This is the second example of an infectious potyviral cDNA driven by a CaMV promoter for \textit{in vivo} transcription; the first example was reported by Maiss et al. (1992) for plum pox potyvirus. In these experiments and in the case of PVY cDNA reported here, the efficiency of infection appears higher than when \textit{in vitro} transcribed potyviral RNA was inoculated. This may be due to difficulties in obtaining full-length transcripts from approximately 10 kbp templates and to the ability of these large RNAs when rubbed onto plant leaves. Also the production of RNA from the CaMV 35S promoter during transient expression \textit{in vivo} can proceed over several hours, allowing more opportunities for starting a productive infection. The biolistic inoculation procedure used here might further improve the infectivity, since only 0.5 µg of DNA per inoculated leaf can lead to nearly 90% infectivity. In comparison, Maiss et al. (1992) used about 10 µg of plasmid DNA to reach the same level of infectivity. However, other parameters might also be involved in determining infectivity, since the construct and test plants were not identical in both experiments.

The approach developed in this paper will make it possible to perform reverse genetics experiments on the PVY genome. PCR technologies can be used to precisely mutagenize and recombine DNA molecules in a few simple steps, using as primers PCR molecules generated with mutagenic synthetic oligonucleotides or with oligonucleotides containing large 5' extensions (Ho et al., 1989; Higuchi, 1990). Thanks to the large collection of biologically well-characterized virus strains, genome fragments can thus be exchanged between different PVY strains in order to map and identify the determinants of pathogenicity and host range.

Table 1. \textit{Summary of two infection experiments with independently PCR-amplified PVY cDNAs}

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<th>Experiment 1</th>
<th>Experiment 2</th>
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<tr>
<td>Bombarded leaves</td>
<td>8</td>
<td>10</td>
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<tr>
<td>Days in growth chamber</td>
<td>5</td>
<td>10</td>
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<tr>
<td>Inoculated plants/leaf</td>
<td>4/4</td>
<td>4/4</td>
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<td>Infected plants</td>
<td>3/4</td>
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Total infected plants | 15/32 | 30/40

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It is anticipated that the method described in this paper might be useful for the quick generation and analysis of infectious cDNAs from plant and animal viruses with large RNA genomes.

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References


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