

Infectious *in vivo* and *in vitro* transcripts from a full-length cDNA clone of PVY-N605, a Swiss necrotic isolate of potato virus Y

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A full-length cDNA clone of the potato virus Y (PVY) genome was obtained after cloning difficulties in *Escherichia coli* were overcome. These difficulties were mainly due to the expression of the CI gene from upstream prokaryotic promoter-like elements within the PVY genome. To overcome this problem, PVY cDNA was maintained in two subclones which were ligated before infection. A plasmid in which these two fragments were contained could be propagated in some *E. coli* strains but was unstable and yielded only low levels of plasmid DNA. Replacement of the 35S promoter by the SP6 promoter slightly increased the stability of the plasmid and its RNA transcripts were infectious when capped with m⁷G(5')ppp(5')G. Using two inoculation methods (mechanical or biolistic) the cDNA and its RNA transcript proved infectious on three *Nicotiana* species and on *Solanum tuberosum*.

With its numerous well-characterized strains and isolates, potato virus Y (PVY) is the type member of the genus *Potyvirus* in the family *Potyviridae* (Shukla *et al.*, 1994) and is an important model for studying the molecular biology of the *Potyviridae*. There are reports of infectious *in vitro* transcripts of full-length cDNA clones of several potyviruses (Domier *et al.*, 1989; Riechmann *et al.*, 1990; Gal-On *et al.*, 1991; Dolja *et al.*, 1992; Puurand *et al.*, 1996). Infectious plasmid DNAs with the potyviral cDNA fused to the cauliflower mosaic virus (CaMV) 35S promoter have also been obtained (Maiss *et al.*, 1992; Gal-On *et al.*, 1995; Johansen, 1996).

However, since the first publication of the complete nucleotide sequence of a PVY isolate (Robaglia *et al.*, 1989), there has been no report of an infectious cDNA of this virus having been cloned and maintained in a bacterial vector,

despite numerous attempts (Fakhfakh *et al.*, 1996). These failures may be due to the expression of cytotoxic products from internal cryptic prokaryotic promoters in the viral genome. To avoid this problem Fakhfakh *et al.* (1996) described a one-step, cell-free method to generate a complete cDNA from PVY genomic RNA using RT-PCR. The cDNA thus obtained, although infectious, does not lend itself to further genetic manipulation. As an alternative solution, Johansen (1996) reported that insertion of intron sequences into full-length pea seedborne mosaic virus (PSbMV) genomic cDNA clones stabilized those in *E. coli*. However, the effective insertion sites were not the same in two closely related strains (P1 and P4) of this virus. This observation supports the previous hypothesis (Fakhfakh *et al.*, 1996) that the toxicity of the potyviral genome in bacteria is a result of the simultaneous presence of cryptic promoter element(s), translational initiation site(s) and cytotoxic gene product(s). This approach, however useful, would be more widely applicable if the cytotoxic gene product could be identified because the positions of the promoter elements are accidental and can be different in various potyvirus species, strains and isolates.

In this paper we describe the construction of infectious full-length cDNA of PVY-N605 (a Swiss vein necrotic isolate from potato cv. Bintje in 1976). This cDNA could be propagated stably as two overlapping halves which, after ligation, generated an infectious cDNA copy. Alternatively, it could be propagated as a single piece in *E. coli* SURE strain (Stratagene) or DH10B strain (Gibco-BRL), although instability of the plasmid was observed.

PVY-N605 (kindly provided by P. Gugerli) was maintained in tobacco (*Nicotiana tabacum*) SR1 plants. Virus purification and RNA extraction were performed according to Gugerli (1986). First and second strand PVY cDNA were synthesized using the λ ZAP cDNA synthesis and cloning kit (Stratagene) according to the manufacturer's instructions. The λ ZAP library was screened using ³²P-labelled PCR fragments specific for the 5' or the 3' regions of the PVY genome. We have confirmed that full-length cDNA of PVY could not be cloned directly in standard plasmid vectors or commonly used host strains of *E. coli* (Fakhfakh *et al.*, 1996). However, we show below that it is

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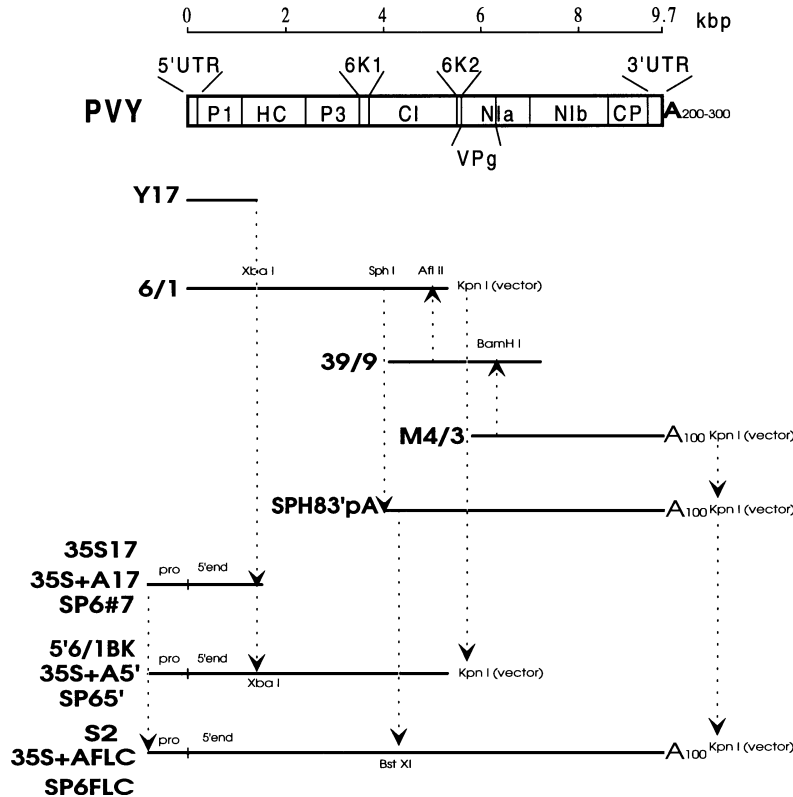


Fig. 1. Construction of the full-length cDNA clone of PVY-N605. The genome organization of PVY is shown at the top. The arrowheads indicate the sites where cDNA fragments were joined by ligation. Pro: 35S or SP6 promoter elements. Clones harbouring the longest fragments – clone 6/1 (5' half of the viral genome, nt 17–5319 in the PVY-N605 sequence, accession no. X97895) and clone M4/3 [3' half, nt 5825–9701 and a 100 nt long poly(A) tail] – were selected for assembly of full-length cDNA. Missing portions of the PVY genome (nt 1–16 and 5320–5824) were obtained by PCR amplification of parts of the double-stranded cDNA using, respectively, primer pairs #1 (5' AATTAACAACACTCAATAC-AACATAAG) and #7 (5' GCCATTACGAGCTGTATTTTC) and #3 (5' ACACTACTTCGCCAATAATCGC) and #9 (5' GCA-TCCATCAAAGGCTGAA). The fragments obtained were cloned into *Sma*I-digested pUC18, generating clones Y17 (nt 1–1492) and 39/9 (nt 4125–7187). For promoter insertions, see text.

possible to produce full-length cDNA by ligation of fragments from partial clones of PVY genome.

The strategy used to produce the full-length cDNA clone of PVY-N605 from partial clones is outlined in Fig. 1. First, elements of the 35S or SP6 promoters were introduced in front of the viral cDNA 5' end in the Y17 clone. The 35S promoter fragment from the pBI221 vector (Clontech) was inserted upstream of the Y17 virus fragment using the *Pst*I and *Bam*HI sites. Unwanted nucleotides between the transcription initiation site and the viral 5' end were removed by PCR (Fig. 2) producing 35S17. The fusion of the SP6 promoter sequence to the 5' end of the cDNA (Fig. 2) was also performed by PCR amplification using Y17 clone as the template and primers #7 (Fig. 1) and SP65'Y (5' AACTGCAG ATTTAGGTGACACTATAGAAATTTAAAACAACTCAA TACAACATAA, promoter region is underlined). The resulting fragment was cloned into pUC18 using the *Pst*I (in the SP65' primer) and the *Xba*I (internal) sites to obtain clone SP6#7. These 5' fragments were then fused to the 6/1 clone to produce constructs 5'6/1BK or SP65', respectively.

Attempts to obtain a near full-length cDNA clone of PVY-N605 by ligation of the appropriate M4/3, 39/9 and 6/1 fragments were unsuccessful, despite the numerous strains of *E. coli* tested, among them ABLE K (Stratagene), which reduces by 10-fold the copy number of pUC18. Plasmids produced after transformation with the ligated fragments had undergone extensive rearrangements (insertions or deletions) in the area

of the CI gene of PVY (data not shown). When part of the CI region (nt 3971–4997) was deleted from the full-length clone without altering the reading frame, or a frameshift mutation was introduced by filling-in the *Bst*XI site at position 4278, the clones became stable yielding 10–15 µg DNA/ml of culture. This suggested that the toxicity is probably associated with the CI gene, which codes for an RNA helicase (Lain *et al.*, 1990), although the participation of other viral products in the toxic effect of PVY cannot be excluded. A frameshift mutation in the P3 region (upstream of the CI) also could stabilize the cDNA clone (data not shown). This may be due to the intrinsic toxicity of P3 and/or the presence of cryptic prokaryotic promoter element(s) in the PVY genome (Fig. 2) allowing the expression of the toxic CI gene.

To avoid the putative toxicity of the CI gene, the PVY genome was split in two parts within the toxic region, an approach used by others to overcome toxicity problems in flaviviruses (Rice *et al.*, 1989; Sumiyoshi *et al.*, 1992) and in beet necrotic yellow vein virus (Quillet *et al.*, 1989). The unique *Bst*XI restriction site in the CI region permitted the joining of the two parts of PVY by *in vitro* ligation. The partial clone from the 5' region (5'6/1BK) included the CaMV 35S promoter and extended to position 5319 in the viral genome. The 3' clone (SPH83'pA) encompassed the viral sequence on the 3' side of position 4065 including a 100 nt poly(A) tail. Both constructs proved stable in DH10B and replicated to high yields (15–20 µg DNA/ml of culture). The *Pvu*II–*Bst*XI fragment of

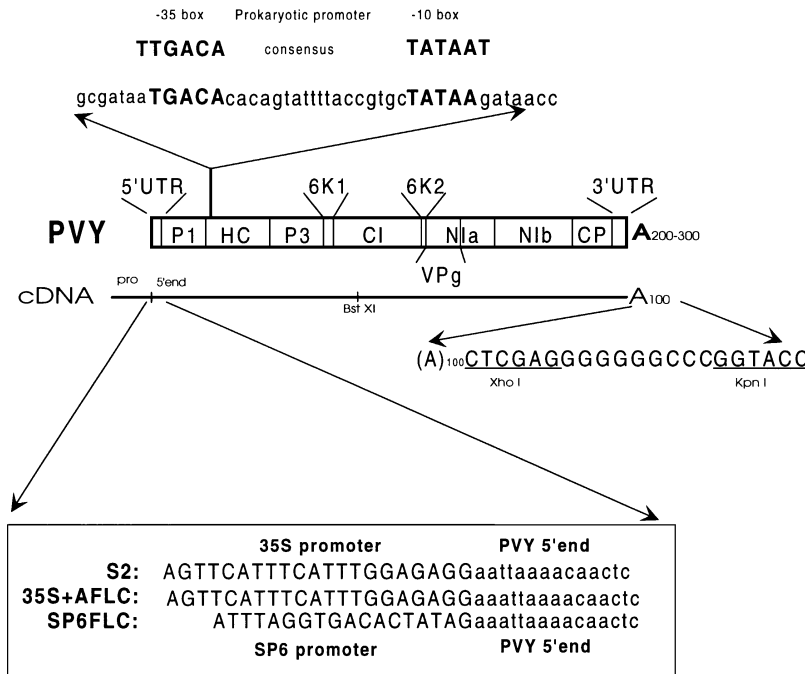


Fig. 2. Characteristic sequence elements of the PVY-N605 cDNA constructs. A region (1156–1197) of the HC gene which displays high homology to the consensus prokaryotic promoter sequence is shown at the top. Identical nucleotides are in bold capital letters. The 5' end of the cDNA of the PVY-N605 genome was fused to the different promoter elements as shown in the insert. Promoter sequences are shown in capital letters and viral sequences are in lower case. The position of the unique *Kpn*I site downstream of the poly(A) tail is also indicated.

the 5'6/1BK construct and the *Bst*XI–*Kpn*I fragment of the SPH83'pA construct, when ligated, were not infectious by mechanical inoculation. However, when using a portable helium flow particle gun (originally an Agracetus Inc. development, now available as Helios gene gun, Bio-Rad), small amounts of ligated DNA (150 ng DNA per leaf) were infectious in three *Nicotiana* species (Table 1).

The biolistic inoculation method, although very efficient, is laborious and expensive compared with mechanical inoculation, although the latter requires more DNA. To obtain higher amounts of cDNA, we decided to pursue our attempts to produce a full-length clone of PVY. The *Bst*XI–*Kpn*I fragment of SPH83'pA was ligated into the plasmid 5'6/1BK digested at the same sites. Upon transformation into the SURE strain of *E. coli*, the resulting clone S2 accumulated to low yields (50–100 ng DNA/ml of culture) and was prone to rearrangement. To avoid extensive rearrangements, it was necessary to amplify the plasmid in small volumes without prior passaging of the inoculum.

After linearization at the 3' *Kpn*I site, S2 DNA proved infectious using the particle gun on both *N. clevelandii* and *N. tabacum* plants (Table 1). Using mechanical inoculation, it was infectious on *N. clevelandii* and *N. benthamiana*, but not on *N. tabacum*. Plants infected with S2 DNA developed typical PVY-N605 symptoms and accumulated PVY RNA with a 2 to 5 day delay compared with wild-type virus.

Primer extension experiments on wild-type RNA and on the RNA of virus derived from the S2 clone (data not shown) revealed the presence, at the 5' end of PVY genome, of an additional adenosine residue not reported previously (Robaglia *et al.*, 1989). This residue, absent in the S2 cDNA, was restored

spontaneously *in planta* during successful infection. However, inclusion of this adenosine in clone S2 (35S + AFLC, Fig. 1) did not significantly increase the infectivity of the DNA (Table 1). For unknown reasons, the undigested DNA proved more infectious after mechanical inoculation on *N. tabacum* than the linear form (Table 1).

In a further attempt to increase the infectivity of cloned PVY-N605 we tested *in vitro* transcripts. The promoter in the 35S + AFLC plasmid was replaced with the SP6 promoter to produce plasmid SP6FLC (Fig. 1), which replicated with slightly increased yields in bacteria (0.5 and 0.8 µg DNA/ml of culture). This plasmid was linearized with *Kpn*I before *in vitro* transcription by SP6 RNA polymerase (Boehringer), with or without inclusion of the cap analogue m⁷G(5')ppp(5')G (Pharmacia) as described by Chapman *et al.* (1992). Only capped *in vitro* transcripts were infectious on *N. clevelandii*, *N. benthamiana* and *N. tabacum* following mechanical inoculation. Infection was more efficient with transcripts than with DNA inoculation, especially on *N. benthamiana* and *N. tabacum* (Table 1). No infection resulted from mechanical inoculation of PVY cDNA or RNA transcript on *Solanum tuberosum* cv. Bintje. Biolistic delivery overcame this problem, although the infection efficiency remained low (Table 1).

Symptom development with the transcript inoculum was slower than with wild-type virus. This delay was not a property of the progeny virus recovered from the transcript- or DNA-inoculated plants. Progeny virus produced symptoms indistinguishable from wild-type PVY-N605. RNA blot hybridization showed accumulation of viral RNAs of similar molecular size in both wild-type and progeny virus (data not shown). In addition, the viral coat protein, as demonstrated by

Table 1. Summary of infection experiments with the different constructs generated from the full-length PVY-N605 cDNA

One leaf per plant was inoculated with the amount of DNA or RNA indicated in parentheses. Successful infections are shown as a percentage of the number of plants inoculated. In parentheses: number of plants showing symptoms (infected)/number of plants inoculated. N. clev.: *Nicotiana clevelandii*, N. bent.: *N. benthamiana*, N. tab.: *N. tabacum* cv. SR1, S. tub.: *Solanum tuberosum* cv. Bintje. N.D., not determined.

Name of the construct / mode of inoculation	Test plants			
	N. clev. (inf/inoc)	N. bent. (inf/inoc)	N. tab. (inf/inoc)	S. tub. (inf/inoc)
5'6/1BK & SPH83'pA ligated / mechanical (1µg DNA/leaf)	N.D.	N.D.	0% (0/12)	N.D.
5'6/1BK & SPH83'pA ligated / particle gun (150ng DNA/leaf)	36% (4/11)	100% (5/5)	67% (4/6)	N.D.
S2 digested / mechanical (10µg DNA/leaf)	83% (5/6)	33% (2/6)	0% (0/29)	0% (0/6)
S2 digested / particle gun (150ng DNA/leaf)	75% (12/16)	N.D.	50% (1/2)	N.D.
35S+AFLC digested / mechanical (10µg DNA/leaf)	70% (7/10)	25% (1/4)	0% (0/10)	0% (0/6)
35S+AFLC undigested / mechanical (10µg DNA/leaf)	56% (9/16)	50% (5/10)	42% (10/24)	N.D.
35S+AFLC undigested / particle gun (100ng DNA/leaf)	100% (5/5)	N.D.	64% (9/14)	33% (3/9)
SP6FLC RNA (capped) / mechanical (1µg RNA/leaf)	75% (9/12)	100% (5/5)	75% (9/12)	0% (0/5)
SP6FLC RNA (capped) / particle gun (100ng RNA/leaf)	100% (5/5)	N.D.	100% (5/5)	30% (3/10)

ELISA, was produced at the same level as in a wild-type infection and the virus particles had normal morphology (data not shown).

In conclusion, the identification of a cytotoxic region within the PVY genome has allowed us to develop a strategy whereby the virus genome could be stably maintained in bacteria as two overlapping subclones and subsequently ligated to produce an infectious cDNA, provided that biolistics is used for inoculation. We also present an alternative strategy in which mechanical inoculation is feasible, but which requires a full-length cDNA clone. In this approach, *in vitro* transcripts consistently resulted in a better infectivity than cDNA

inoculations. Finally, the choice of the test plant species is critical in such strategies.

Derivatives of these constructs are currently being used with transgenic plants expressing viral sequences of the same isolate (Farinelli & Malnoë, 1993; Malnoë *et al.*, 1994) in a risk assessment programme. The experiments will allow us to examine the effects and frequency of recombination between viral genomes and transgenes expressed in host plants, data which are important for the evaluation of the risks associated with the exploitation of genetically engineered organisms (Falk & Bruening, 1994).

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