

Cauliflower mosaic virus 35S promoter-controlled DNA copies of cowpea mosaic virus RNAs are infectious on plants

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Clones have been constructed that contain full-length cDNA copies of cowpea mosaic virus RNA1 and RNA2, downstream of the cauliflower mosaic virus 35S promoter. The clones, when linearized downstream of the viral sequences, give rise to cowpea mosaic virus-like

symptoms when inoculated onto cowpea plants. Viral RNA and virions can be detected in the inoculated plants, demonstrating that the clones are directly infectious.

The genome of cowpea mosaic virus (CPMV), type member of the comovirus group of plant viruses, consists of two messenger-sense RNA molecules termed RNAs 1 and 2 (also known as B and M RNA, respectively) which are separately encapsidated in icosahedral capsid structures (for a review, see Goldbach & van Kammen, 1985). The study of CPMV at the molecular level has been facilitated by the availability of genome-length cDNA copies of the RNAs that are linked to a RNA polymerase promoter sequence. These can be used to generate *in vitro* transcripts that are infectious when inoculated into protoplasts or onto whole plants (Vos *et al.*, 1988; Eggen *et al.*, 1989; Holness *et al.*, 1989; Dessens & Lomonosoff, 1991). In every case, however, the infectivity of the transcripts is significantly lower than that of natural virion RNAs, probably as a result of the presence of non-viral residues at the termini of the transcripts. Difficulties may also be caused by exposure of the transcripts to degradative agents during inoculation. For this reason the transcripts are usually stabilized by capping their 5' ends, but this is an inefficient, costly and time-consuming process.

Recently, the construction of directly infectious cDNA clones of brome mosaic virus RNAs 1, 2 and 3 (Mori *et al.*, 1991), beet necrotic yellow vein virus RNAs 3 and 4 (Commandeur *et al.*, 1991), pea early browning virus RNAs 1 and 2 (MacFarlane *et al.*, 1992), tomato mosaic virus RNA (Weber *et al.*, 1992) and plum pox virus RNA (Maiss *et al.*, 1992) have been reported. These constructs make use of the cauliflower mosaic virus (CaMV) 35S promoter sequence linked to the 5' ends of the viral cDNAs to generate infectious transcripts in the plant. This technique potentially overcomes some of the problems encountered with the use of transcripts generated *in vitro*. To test whether this approach is applicable to CPMV, clones

containing full-length cDNAs of RNAs 1 and 2 downstream of the CaMV 35S promoter were constructed and tested for their ability to infect cowpea plants.

A modified CaMV 35S promoter sequence was excised from plasmid pCaP35J (Yamaya *et al.*, 1988) with *Bam*HI (filled in with Klenow polymerase after digestion) and *Pst*I and was cloned into either *Hind*III- (filled in with Klenow polymerase after digestion)/*Pst*I-digested M13mp18 replicative form (RF) DNA or similarly treated pUC18 plasmid DNA, to give mp18-35S and pUC18-35S, respectively. By doing so, the positions of restriction sites flanking the promoter were changed so that no sites were left upstream of the promoter sequence and all the mp18 cloning sites from *Pst*I to *Eco*RI were present downstream (see Fig. 1). The promoter sequence contains a *Sst*II restriction site at the 5' end and a *Stu*I site at the 3' end. Digestion with the latter enzyme exposes the 3' end of the promoter sequence for direct blunt-end ligation of foreign sequences.

Full-length first strand cDNA to RNA2 was synthesized with Superscript reverse transcriptase (Gibco BRL) using pdT₁₂₋₁₈ as a primer. Second strand synthesis was performed as described by Lomonosoff *et al.* (1982) using pd(TATTAAATCTTAATAGG), corresponding to the first 18 nucleotides of RNA2. Full-length double-stranded DNA copies of CPMV RNA1 were synthesized as described in Dessens & Lomonosoff (1991). The full-length cDNAs were digested with either *Bam*HI (position 1505 of RNA2; van Wezenbeek *et al.*, 1983) or *Sst*I (position 2301 of RNA1; Lomonosoff & Shanks, 1983) and the 5' end fragments were cloned into *Stu*I/*Bam*HI-digested or *Stu*I/*Sst*I-digested mp18-35S, to give mp18M-35S and mp18B-35S, respectively (for details of the cloning strategy, see Fig. 1). Correct fusion of the 5' ends to the 35S promoter sequence was

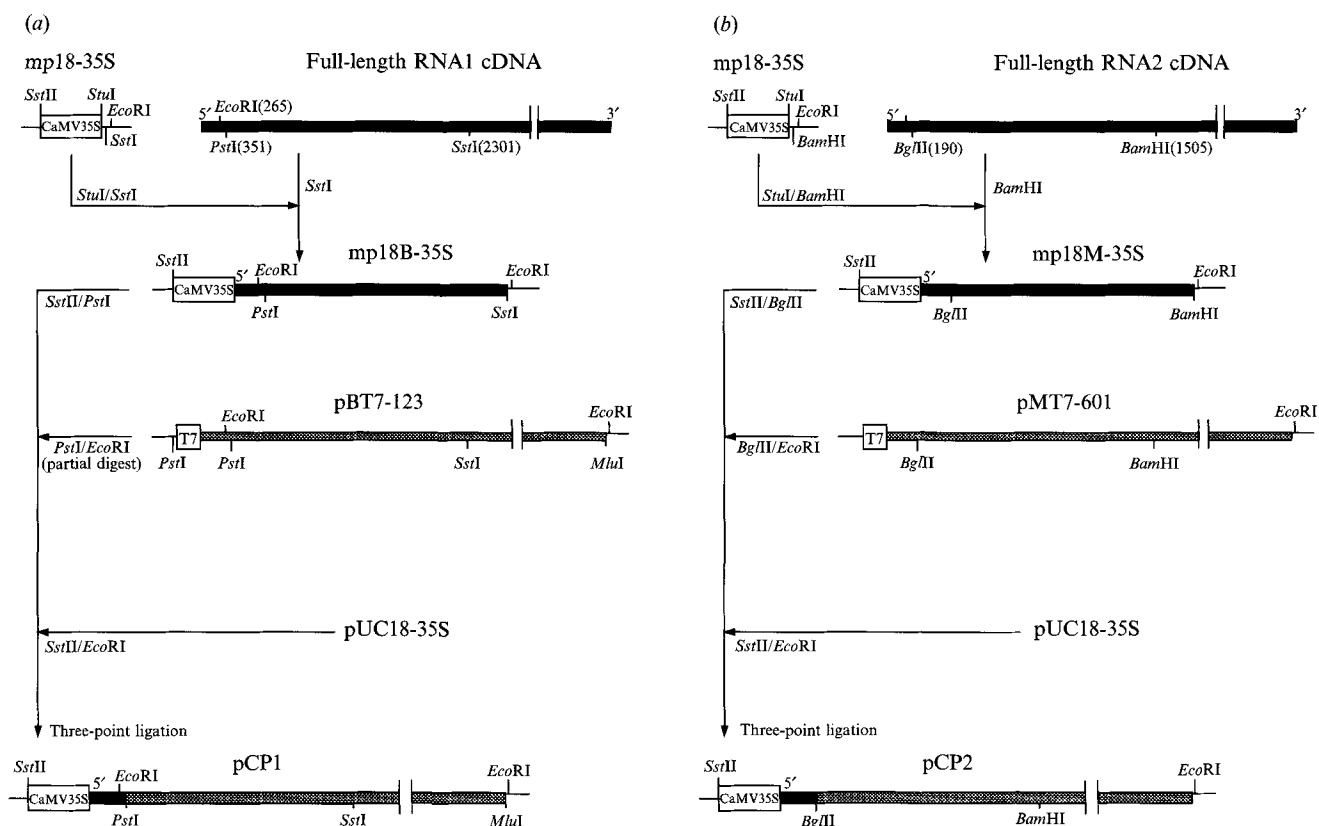


Fig. 1. Schematic diagram of the cloning strategies for constructing pCP1 (a) and pCP2 (b). cDNA to CPMV RNAs is represented by black bars, DNA taken from full-length cDNA clones of RNA1 (pBT7-123) and RNA2 (pMT7-601) is represented by shaded bars. Various restriction sites used in cloning are indicated.

confirmed by sequence analysis of single-stranded M13 DNA (data not shown). RF DNA from mp18M-35S and mp18B-35S was digested with *Sst*II and either *Bgl*II (position 190 of RNA2) or *Pst*I (position 351 of RNA1) and the 515 bp *Sst*II/*Bgl*II and 676 bp *Sst*II/*Pst*I fragments (containing the 35S promoter sequence plus 5' ends of the cDNAs) were isolated. The rest of the RNA2 sequence was excised from pMT7-601, a derivative of pPMM2902 (Holness *et al.*, 1989) in which the *Escherichia coli* promoter is replaced with a T7 promoter (Rohll *et al.*, 1993), with *Bgl*II and *Eco*RI (downstream of the RNA2 sequence; see Fig. 1), and the 3298 bp *Bgl*II/*Eco*RI-fragment was isolated. The rest of the RNA1 sequence was excised from pBT7-123 (Dessens & Lomonosoff, 1991) with *Pst*I and *Eco*RI (partial digest since an *Eco*RI is also present downstream of the RNA1 sequence; see Fig. 1) and a 5569 bp *Pst*I/*Eco*RI fragment was isolated. Subsequently, the two fragments containing RNA2 sequences and the two fragments containing RNA1 sequences were cloned into *Sst*II/*Eco*RI-digested pUC18-35S by three-point ligations, to give clones pCP2 and pCP1, respectively. Thus, these clones contained the genome-length viral sequences downstream of the CaMV

35S promoter in pUC18 (Fig. 1). Transcripts derived from these clones in the plant should have no non-viral nucleotides at their 5' ends, in contrast to *in vitro* transcripts derived from pBT7-123 and pMT7-601 which have a single non-viral G residue as their first nucleotide.

To investigate whether a combination of pCP1 and pCP2 DNA can infect cowpea plants, DNA was purified by centrifugation through ethidium bromide/caesium chloride gradients and was linearized with *Mlu*I and *Eco*RI, respectively. Inoculation of 10-day-old cowpea (*Vigna unguiculata* L.) seedlings was carried out by dusting the primary leaves with carborundum powder followed by manually abrading with 50 μ l aliquots of an inoculum consisting of a mixture of various amounts of linearized pCP1 and pCP2 DNA dissolved in water. One leaf per plant was inoculated and the plants were maintained in the greenhouse at 25 $^{\circ}$ C. When the inoculum consisted of 10 μ g of each of the linearized plasmids all four inoculated plants developed symptoms by 3 weeks post-inoculation. The symptoms were typical of a CPMV infection, consisting of chlorotic lesions on the inoculated leaves and systemic mosaic on the upper

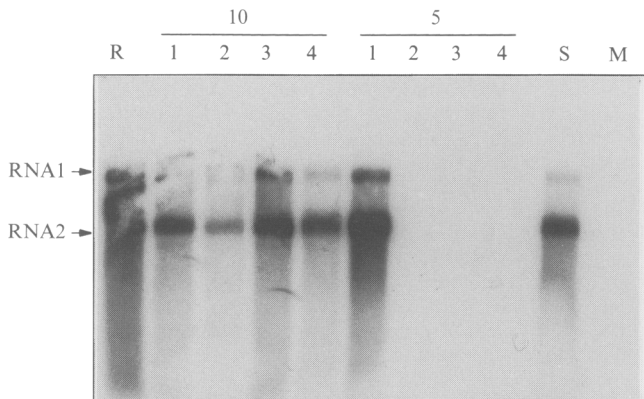


Fig. 2. Northern blot analysis of nucleic acid extracted from cowpea plants inoculated with linearized pCP1 and pCP2 DNA (lanes marked 5 and 10). Samples were taken 3 weeks post-inoculation. Four plants were inoculated with 10 μ g of each DNA (lanes marked 10) and four plants with 5 μ g of each DNA (lanes marked 5). Lane R contains nucleic acid extracted from a plant inoculated with a mixture of 5 μ g each of capped transcripts derived from pBT7-123 and pMT7-601; lane S contains nucleic acid extracted from a plant inoculated with sap from a DNA-inoculated and infected plant; lane M contains nucleic acid from a mock-inoculated plant. The filter was probed for CPMV RNAs 1 and 2. Positions of RNAs 1 and 2 are indicated on the left-hand side of the gel.

leaves. When smaller amounts of DNA were applied (5 or 2 μ g of each plasmid) only one in four plants developed symptoms in each case.

To verify that the symptoms noted above were due to infection of the plants with CPMV, total nucleic acid was isolated from the plants 3 weeks post-inoculation using the method of Verwoerd *et al.* (1989). The RNA was fractionated on formaldehyde/MOPS-containing agarose gels (Lehrach *et al.*, 1977), transferred to a Hybond-N membrane (Amersham) and subjected to Northern blot analysis using 32 P-labelled probes prepared according to the method of Feinberg & Vogelstein (1984). The RNA2-specific probe consisted of nucleotides 482 to 2211 of RNA2 and the RNA1-specific probe consisted of nucleotides 2301 to 3858 of RNA1. The Northern blot analysis revealed that all the leaves which had been inoculated with 10 μ g each of pCP1 and pCP2 contained CPMV RNAs 1 and 2 (Fig. 2). When the inoculum consisted of only 5 μ g of each plasmid, only those leaves which developed symptoms (one in four) revealed the presence of the CPMV-specific RNAs. The same was true of leaves inoculated with 2 μ g of each plasmid (data not shown). Indeed, throughout this work the development of symptoms was found to be an entirely reliable guide to the occurrence of an infection. The level of CPMV-specific RNA found in extracts from leaves infected with pCP1 and pCP2 was similar to that found in leaves which had been infected with a mixture of 5 μ g each of GpppG-capped transcripts

derived from plasmids pBT7-123 and pMT7-601 (Fig. 2, lane R). Inoculation of healthy cowpea plants with sap from a pCP1/pCP2-inoculated and infected plant resulted in infection (Fig. 2, lane S), demonstrating that virus derived from pCP1 and pCP2 can be successfully passaged.

Leaf tissue was analysed for the presence of virus particles by the 'leaf-dip' method (Hitchborn & Hills, 1965). CPMV-like particles, approximately 28 nm in diameter, were detected in the DNA-inoculated leaves (data not shown), demonstrating that the RNAs are encapsidated. The presence of visible lesions on the inoculated leaves and the appearance of mosaic symptoms on the upper leaves supported this notion since encapsidation is thought to be a prerequisite for both cell-to-cell and long distance movement of CPMV (Wellink & van Kammen, 1989).

The constructs used in this study do not contain a polyadenylation signal downstream of the viral sequence, in contrast to the 35S-controlled clones constructed by Mori *et al.* (1991), Commandeur *et al.* (1991), MacFarlane *et al.* (1992), Weber *et al.* (1992) and Maiss *et al.* (1992). Instead, the clones used in this study are linearized downstream of the viral sequence, thus preventing the synthesis of transcripts longer than the genome. The sites used for linearization were identical to those used when making *in vitro* transcripts from pBT7-123 and pMT7-601 (Dessens & Lomonossoff, 1991; Rohll *et al.*, 1993). The RNA1 and RNA2 transcripts produced either *in vitro* or in the plant are therefore expected to have zero and seven non-viral nucleotides, respectively, downstream of the poly(A) tract. Inoculation with 10 μ g of non-linearized DNA from each plasmid did not result in infection, judging from the lack of symptom formation (data not shown), confirming the importance of preventing the synthesis of over-size transcripts.

Efficient inoculation with the DNA constructs requires 10 μ g of each (ds) DNA per plant. For efficient inoculation with capped RNA transcripts, 5 μ g of each RNA is used per plant, which is approximately equivalent to 5 μ g of the transcribed plasmid DNA (Dessens & Lomonossoff, 1991). Thus, the total amount of plasmid required for direct inoculation with DNA is about twice that required if transcripts are made first. However, since the direct use of DNA avoids all the problems and expense of carrying out *in vitro* transcription it still has a considerable advantage over the use of transcripts. Furthermore the clones may prove useful for transient expression studies in protoplasts. Hence the system reported here should facilitate the analysis of the CPMV replication cycle and contribute towards a more rapid understanding of the molecular biology of the virus.

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