Efficient infection from cDNA clones of cucumber mosaic cucumovirus RNAs in a new plasmid vector

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Full-length cucumber mosaic cucumovirus (CMV) cDNAs were cloned into a new plasmid vector containing a modified plant virus promoter designed to transcribe the inserted sequence from its first nucleotide. cDNA copies of CMV strain Q (Q-CMV) genomic RNAs 1, 2 and 3 cloned into this vector were infectious when inoculated together, producing symptoms indistinguishable from those caused by wildtype Q-CMV infection. The infectivity of the clones could be substantially increased by excision of the viral insert together with the transcriptional promoter and terminator prior to inoculation. A diagnostic but silent mutation was introduced into the RNA 2 cDNA and found to be stably maintained in viral infection, allowing distinction of the recombinant virus from native contaminants. The infectious cDNA clones supported the replication of CMV satellite RNA when co-inoculated with biologically active Q-CMV satellite RNA transcripts. Using the infectious cDNAs described, it was found that a newly-identified overlapping gene (2b) encoded by Q-CMV RNA 2 was not essential for either systemic viral infection of Nicotiana glutinosa or replication of the satellite RNAs.

Infectious full-length cDNA clones are an important tool for investigating the molecular biology of plant RNA viruses. Such clones have been obtained for a large number of unrelated RNA viruses (Boyer & Haenni, 1994). However, only a few of these are truly infectious cDNAs, rather than DNA templates from which infectious RNA molecules must be transcribed (Boyer & Haenni, 1994, and references therein). This is despite the fact that the first infectious clones of RNA viruses were active as cDNAs (e.g. Taniguchi et al., 1978), and that infectious cDNAs have several advantages over infectious RNA transcripts. Firstly, DNA is more stable than RNA and therefore easier to use as inoculum. Secondly, the expense and technical difficulties associated with in vitro transcription are circumvented by using infectious cDNA clones.

We report here the construction of a new and convenient cloning vector (pCass; see Fig. 1) which contains a cDNA expression cassette consisting of the 3SS transcription promoter and terminator signals from cauliflower mosaic virus (CaMV). The promoter contains a modification at the transcription start site which allows the viral sequence of interest to be transcribed in planta with no extraneous vector sequences at the 5' end. We demonstrate the utility of this vector in the construction of full-length infectious cDNA clones of cucumber mosaic cucumovirus strain Q (Q-CMV).

Virions and viral RNAs of Q-CMV (Francki et al., 1966) were purified as described previously (Ding et al., 1994). First-strand cDNA synthesis from all three genomic RNAs was primed from oligonucleotide SD6 (5' ACGGATCCTGGTCTCCTTATGGAGAACCTGTGG 3'), which is complementary to the 3' terminal 25 nt sequence common to all three genomic RNAs, and contains a BamHI site at the 5'-end. cDNA synthesis was performed on total virion RNAs using avian myeloblastosis virus (AMV) reverse transcriptase (Promega) under the conditions suggested by the manufacturer with the following modifications: the cDNA synthesis was initially carried out at 37°C for 5 min, and then continued at 50°C for a further 30 min. Distinct bands of full-length single-stranded cDNAs corresponding to Q-CMV RNAs 1, 2, 3, 4 and 4A (Ding et al., 1994) were clearly visible after agarose gel electrophoresis and ethidium bromide staining (data not shown). The mixed first-strand cDNAs were amplified in separate polymerase chain reactions (PCR) using methods similar to those of Boccard & Baulcombe (1992) with one of the following sets of primers: SD6 and SD8 (5' ACGGATCCTGGTCTCCTTATGGAGAACCTGTGG 3', homologous to nucleotides 1–23 of RNA 1) for amplifying RNA 1 cDNA; SD6

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Fig. 1. Structures of the cloning vector pCass and infectious full-length cDNA clones of Q-CMV RNAs 1, 2 and 3 (pQCD1, pQCD2 and pQCD3). pCass and pQCD2/pQCD3 were constructed in pSP72 whereas pQCD1 was constructed in pUC19. Boxed P and T represent CaMV 35S promoter and terminator, respectively. The StuI site of pCass is methylation sensitive; thus it is necessary to propagate pCass in dcm- strains of E. coli, such as JM110. BamHI", a short polylinker of 5' BamHI-SmaI-PstI-EcoRI 3'; BamHI", 5' BamHI-SpeI-XbaI-NotI-SacII-SacI-EcoRI 3'; PvuII, 5' XhoI-PvuII-HindIII-Sphl-PstI 3'; SalI", 5' XbaI-SalI-PstI-SphI-HindIII 3'.

We constructed a new vector (pCass) to facilitate the cloning of full-length infectious cDNAs of plant RNA viruses (Fig. 1). This vector utilizes the 35S promoter of CaMV modified such that viral sequences can be inserted at the +1 transcription site, according to a scheme originally devised by Yamaya et al. (1988). The 35S promoter was amplified from plasmid pRT103 (Töpfer et al., 1987) by PCR using oligonucleotide CaMV3StuI (5' AGGCCTCTCCAATGAAATGAAC 3'; complementary to the 3' end of the promoter and containing a StuI recognition sequence at the transcriptional initiation site) and the M13 universal sequencing primer (USP). The PCR product of 480 nt was digested with PstI and cloned into PstI-smal-digested pSP72 (Promega) to create p35SPROM. A transcriptional termination signal was obtained from the plasmid pJIT60, a pUC-based plasmid containing the 35S RNA transcriptional promoter and terminator sequences from CaMV (a gift of Dr I. B. Dry, CSIRO Division of Horticulture, Glen Osmond, Australia). To obtain the minimal sequence necessary for termination (Dr I. B. Dry, personal communication), the plasmid was digested with EcoRI and BglII to release a 240 nt fragment which was ligated into EcoRI-BglII-restricted p35SPROM to produce pCass (Fig. 1). Thus, the 35S promoter and terminator of CaMV flank a short polylinker of four restriction enzyme recognition sites (StuI, KpnI, SacI and EcoRI). Sequences blunt-cloned into the StuI site of pCass will contain no vector sequences after in vivo transcription. This is important because 5' non-viral extensions often substantially decrease or even abolish infectivity, whereas 3' additions are more easily tolerated; no commercially available vector offers these features (Boyer & Haenni, 1994).

A general method for constructing infectious full-length cDNA clones into pCass was developed. The 5'-terminal portions of Q-CMV RNAs 1, 2 and 3 (from pQCR1, pQCR2 and pQCR3) were subcloned into pBluescript SK(+) (Stratagene) prior to PCR amplification of the viral sequences using Vent DNA Polymerase (New England Biolabs), which gives blunt ends; details for pQCR3 are given as an example (Fig. 2). The PCR products were digested with the appropriate restriction enzyme (SacI for RNAs 1 and 2; EcoRI for RNA 3) at the 3' end and then cloned into StuI and SacI-or EcoRI-digested pCass to give plasmid 5'QCD1, 5'QCD2 and 5'QCD3 (Fig. 2). This strategy allows the 5'-end nucleotide of the in planta RNA transcript to be precisely defined by that of the 5' PCR primer sequence.
The remaining viral sequences (nucleotides 492 (SacI–2197 of RNA 3 and nucleotides 581 (ApaI)–3035 of RNA 2) were assembled into the above recombinant plasmids (p5’QCD2 and p5’QCD3) to produce pQCD2 and pQCD3.

While this strategy was successful in cloning the cDNAs of RNAs 2 and 3, repeated attempts failed to assemble the remaining sequence of RNA 1 (nucleotides 337 (NcoI)–3389) into p5’QCD1. We found that the full-length cDNA from RNA 1 was stably cloned into Smal- and BamHI-restricted pUC19 (pQCR1, see above). Therefore, the HindIII–NcoI fragment (containing the 35S promoter/5’-terminal sequence) from p5’QCD1 and the BamHI–BglII fragment (containing the 35S terminator) from pQCD3 were cloned into pCDR1 to give the full-length cDNA clone of RNA 1, pQCD1 (Fig. 1). pQCD1, pQCD2 and pQCD3 have been stably maintained in E. coli strain DH5α for over 2 years.

Plasmid DNAs of the three clones (pQCD1, pQCD2 and pQCD3) were purified by Superose 6 gel filtration chromatography (Skingle et al., 1990). The sequence of all clones was partially determined at the 5'- and 3'-terminal regions (200–300 nt) to confirm the integrity of the promoter fusions, and to assess the extent of nucleotide changes from the published sequences (Rezaian et al., 1984, 1985; Davies & Symons, 1988). No variations were observed in the sequences of pQCD1, pQCD2 and pQCD3 that correspond to the 5’ untransformed regions of RNAs 1, 2 and 3. However, a number of variations were observed in regions of pQCD1 and pQCD2 that correspond to the 3’ untransformed regions of RNAs 1 and 2. Changes to pQCD1 included substitutions (C 3259 to G) and a C insertion between nucleotides 3219 and 3220. The differences between the nucleotide sequence of pQCD2 and the published RNA 2 sequence (Rezaian et al., 1984) include one substitution (C 2905 to G in pQCD2), two deletions (A 2864 and G 2865) and two insertions (C between nucleotides 2864 and 2865, and between nucleotides 2889 and 2890). All of these changes present in pQCD2 were previously observed in the sequence of a cDNA clone of Q-CMV RNA 4A, a subgenomic RNA of RNA 2 (Ding et al., 1994). Importantly, the substitutions at nucleotide 3259 of RNA 1 and nucleotide 2905 of RNA 2 restore the previously non-base paired C–U at the B arm of the tRNA-like structures of RNAs 1 and 2 to a G–U base pair (Palukaitis et al., 1992). This sequence change also creates a recognition site for KpnI, which we confirmed.
Short communication

(a) (b)

1 2 3 4 5

6 7 8 9 10

sat

Fig. 3. Northern blot analysis of fractionated total RNAs extracted from healthy *N. glutinosa* plants (lanes 1 and 5) or from plants inoculated with Q-CMV virions (lanes 3 and 6), with pQCD1, pQCD2 and pQCD3 (lanes 4 and 8), or co-inoculated with Q-CMV sat-RNA transcripts and either Q-CMV virions (lane 7) or double digested pQCD1, pQCD2 and pQCD3 (lane 9). Purified viral RNAs were loaded in lane 2 and full-length sat-RNA transcripts (see text) used for plant inoculations were loaded in lane 10. The Hybond-N* membranes were hybridized with Q-CMV genomic RNA-specific (a) or Q-CMV sat-RNA specific (b) probes. The fastest migrating band (labelled as 5, lanes 2, 3 and 4) most likely corresponds to RNA 5, described previously (Peden & Symons, 1973; Palukaitis et al., 1992). The identities of the two bands between RNAs 3 and 4 (lanes 3 and 4) are under investigation.

Table 1. Infectivity of the cloned cDNAs of Q-CMV and sat-RNA transcripts

<table>
<thead>
<tr>
<th>Inoculum</th>
<th>Circular</th>
<th>Linearized</th>
<th>Double-digested</th>
</tr>
</thead>
<tbody>
<tr>
<td>pQCD1 + pQCD2 + pQCD3*</td>
<td>6/20</td>
<td>4/10</td>
<td>14/15</td>
</tr>
<tr>
<td>pQCD1 + pQCD2A2b + pQCD3*</td>
<td>0/15</td>
<td>ND</td>
<td>8/10</td>
</tr>
<tr>
<td>pQCD1 + pQCD2 + pQCD3* + Q sat-RNAt</td>
<td>0/10</td>
<td>ND</td>
<td>3/5</td>
</tr>
</tbody>
</table>

* Plasmid DNA (5 μg: circular or digested) from each of the three Q-CMV cDNA clones and all in 10 μl water was inoculated onto every *N. glutinosa* plant dusted with carborundum.
† As for (*) plus Q-CMV sat-RNA transcripts obtained from 1 μg Smal-linearized pUC19SAT plasmid DNA.
‡ Number of plants that became infected with Q-CMV over the total number of plants inoculated.
§ In two separate experiments using 10 μg plasmid DNA of each of the three cDNA clones for each plant in 10 μl water, two out of the ten plants inoculated became infected.
∥ Number of plants that became infected with sat-RNA over the total number of plants inoculated.
ND, Not determined.

by digestion of pQCD1, pQCD2 and pQCD3 with this enzyme (data not given).

To test for infectivity, purified circular plasmid DNAs of pQCD1, pQCD2 and pQCD3 were mixed in equal amounts (w/w; 5 μg per plasmid DNA for each plant in 10 μl water) and inoculated on plants which had been kept in the dark for 24 h. The mixture of pQCD1, pQCD2 and pQCD3 systemically infected *Nicotiana glutinosa* at a variable frequency of 10–50% (Table 1), producing symptoms indistinguishable from those of infection by purified virions of Q-CMV. Total RNAs were purified (Verwoerd et al., 1989) from healthy and systemically infected leaves and subjected to Northern blot analysis, using a 32P-labelled RNA probe with sequence complementary to the 3' terminal 340 nt of RNA 2. This RNA 2-derived probe is capable of detecting all Q-CMV RNAs because the 3' terminal sequences (about 300 nt) of these RNAs are highly homologous (Symons, 1979). Viral genomic RNAs (1, 2 and 3) and subgenomic RNAs (4 and 4A) were detected both in cDNA-inoculated (Fig. 3a, lane 4) and virus-inoculated (lane 3) *N. glutinosa* plants. Western blot analysis also demonstrated the accumulation of Q-CMV coat protein in the cDNA-inoculated plants (data not shown), using a rabbit antiserum raised against Q-CMV coat protein (a gift from Roderick Bonfiglioli, Waite Institute, University of Adelaide, Glen Osmond, Australia). The plasmid DNAs pQCD1, pQCD2 and pQCD3 were also infectious on cucumber (*Cucumis sativus* cv. Green Gem) and cowpea (*Vigna unguiculata* cv. Blackeye) producing symptoms similar to those caused by infection from Q-CMV virions (data not shown). These data demonstrate the infectivity of the clones and confirm the usefulness of PCR in the construction of full-length cDNA clones of RNA viruses (viz. Hayes & Buck, 1990; Boccard & Baulcombe, 1992).

In an attempt to improve the frequency of infection obtained above, we experimented with restriction enzyme digestion of the cDNA clones prior to inoculation. Linearization of the plasmids did not significantly improve the efficiency of infection relative to that achieved with closed circular DNA (Table 1). However,
if the viral expression cassettes (consisting of full-length viral cDNA flanked by the 35S promoter and terminator) were excised from the vector by restriction enzyme digestion (NdeI and SalI for pQCD1; PvuII and BgII for pQCD2 and pQCD3; see Fig. 1), marked increases in infectivity were obtained. In three independent experiments comprising inoculation of five N. glutinosa plants, 14/15 plants became infected after double digestion of the plasmid DNAs (Table 1). Therefore, the strategy of excising the viral expression cassettes of the infectious plasmid DNAs prior to plant inoculation has since been used routinely, and subsequent experiments have confirmed the high infectivity of the DNAs treated in this matter (data not shown).

For the use of these clones in reverse genetics, it is essential that they are sufficiently infectious so that in vitro-created mutants (which are likely to exhibit reduced infectivity) are also infectious. To test this, we abolished the open reading frame (ORF) of a newly identified overlapping gene (2b) of Q-CMV (Ding et al., 1994), which resides on RNA 2. A mutant cDNA clone (pQCD2Δ2b) was constructed in which a stop codon was introduced by single nucleotide substitution (A242° to T) into ORF 2b, three codons after its initiation codon (S. W. Ding and others, unpublished data). None of 15 N. glutinosa plants inoculated in three independent experiments with pQCD2Δ2b DNA (plus wild-type pQCD1 and pQCD3) produced an infection. Inoculations with double the amounts of plasmid DNAs (30 μg plasmid DNAs in 10 μl water for each plant) resulted in a low infection frequency (2/10; Table 1). However, when plasmid DNAs with excised viral expression cassettes were used as inocula, 8/10 plants became infected (Table 1). Progeny analysis, using reverse transcription and PCR (RT–PCR) followed by cloning and complete sequence determination of the 3’-terminal 682 nt of RNA 2, found no revertant or pseudorevertant from N. glutinosa plants systemically infected by the ORF2b-deletion mutant. Thus, we conclude that the 2b gene is not essential for the systemic viral infection of N. glutinosa.

Furthermore, we also found that pQCD1, pQCD2 and pQCD3 in the closed circular form could not function as helper for the replication of CMV satellite RNA (sat-RNA) without prior excision of the viral expression cassettes (Table 1; also see below). These data also indicate the importance of increased infectivity obtained by excision of the viral expression cassette prior to inoculation.

It is desirable to distinguish wild-type Q-CMV infection from virus progeny derived from the infectious cDNA clones. Therefore, we introduced a diagnostic but silent mutation (T7213 to A) into pQCD2 by PCR mutagenesis (Higuchi et al., 1988), in order to create an EcoRI recognition site. When co-inoculated with wild-type pQCD1 and pQCD3, the mutant clone (designated pQCD2-EcoRI) was infectious on N. glutinosa, producing symptoms indistinguishable from those caused by wild-type clones (pQCD1, pQCD2 and pQCD3). The viral progeny RNA obtained from inoculation with the above mutated clone (pQCD2-EcoRI, plus pQCD1 and pQCD3) was analysed by RT–PCR, and then digested to completion with EcoRI. The results indicated that the progeny RNA 2 contained the introduced mutation; this provides strong evidence that it was derived from the inoculated plasmid DNA (data not shown).

An important biological property of CMV is its capacity to act as a helper virus for CMV sat-RNA (Francki, 1985). To test the replication of Q-CMV sat-RNA by the pQCD clones, a plasmid DNA clone containing a full-length cDNA copy of Q-CMV sat-RNA (pUC19SAT; P. Q. Thomas & R. H. Symons, unpublished data) was constructed from a previously described full-length cDNA clone (pQSAT; Davies, 1988). SmaI-linearized pUC19SAT was transcribed with T7 RNA polymerase to give a transcript containing only four non-satellite nucleotides, all at the 5’ end. Sat-RNA transcripts thus prepared were inoculated together with the three pQCD wild-type clones with excised viral expression cassettes. Northern blot analysis (Fig. 3b) showed that the sat-RNA transcripts were infectious on N. glutinosa plants co-inoculated with either purified viral particles of Q-CMV (lane 7) or pQCD1, pQCD2 and pQCD3 (lane 9), using 32P-labelled minus-strand sat-RNA transcripts (complementary to the 3’-terminal 112 nt of Q-CMV sat-RNA) as a probe. We also found that sat-RNA was successfully replicated in the plants when the ORF2b-deletion mutant RNA 2 cDNA clone (pQCD2Δ2b) and pQCD1/pQCD3 were used as the helper, indicating that the 2b gene was not required for replication of sat-RNA (data not shown).

In this communication, we have described the construction of infectious full-length cDNAs of Q-CMV using a new transcriptional plasmid vector, pCass. The vector allows convenient cloning of viral cDNA sequences according to a generalized strategy, allowing determination of the 5’ nucleotide of the viral transcript. The infectivity of the cDNA clones was markedly increased by excision of the transcription cassette prior to inoculation. This finding is very important for experiments employing mutation of the viral cDNAs, or for studies exploring the interaction between CMV and its satellite RNA. Low infectivity of closed circular viral cDNA clones driven by the CaMV 35S promoter has also been reported for brome mosaic bromovirus (Mori et al., 1991) and tomato mosaic tobamovirus (Weber et al., 1992). The increase in infectivity obtained by digestion of the infectious clones to release the trans-
criptional cassette described here is similar to the results of Neelaman et al. (1993) who found that cDNA clones of alfalfa mosaic virus were only infectious after release of the viral inserts. We introduced a tagging mutation to the RNA 2 cDNA which distinguishes the recombinant virus from the wild-type, but which does not change any biological property of the virus. The recombinant clones also supported the replication of the Q-CMV satellite RNA. Using this system, we found that the small overlapping gene encoded by Q-CMV RNA 2 was not essential for either systemic viral infection of N. glutinosa or sat-RNA replication. In conclusion, we now have an efficient and convenient system for the application of reverse genetics to the study of cucumoviruses.

The authors wish to thank Dr Neil Shirley for oligonucleotide synthesis and Wendy Winnall for technical help. This work was supported financially by the Australian Research Council Special Research Centre in Basic and Applied Plant Molecular Biology. J. P. R. is the recipient of a Senior Post-Graduate Award from the Grains Research and Development Corporation.

References


