Plasmid vector for cloning infectious cDNAs from plant RNA viruses: high infectivity of cDNA clones of tomato aspermy cucumovirus

Bu-Jun Shi, Shou-Wei Ding† and Robert H. Symons

Department of Plant Science, Waite Institute, University of Adelaide, Glen Osmond, SA 5064, Australia

An improved version of the previously obtained cloning vector pCass was constructed by partially duplicating the 35S promoter used to drive the transient transcription of cloned viral cDNAs. Full-length cDNAs of the three genomic RNAs of tomato aspermy cucumovirus (TAV) cloned in this improved pCass (designated pCass2) gave a 3-fold higher infectivity in two plant species tested than the same cDNAs cloned in pCass1 with only a single 35S promoter. Host range, symptoms, morphology of viral particles and viral progeny RNAs induced by these sets of infectious cDNA clones analysed were identical to those induced by the wild-type virus. A mutant of genomic TAV RNA 3 containing a 163 nt deletion in the 3′ untranslated region was stably maintained in the progeny RNAs, indicating that these cDNA clones may facilitate a study of virus function. This is the first report of infectious cDNA clones of TAV as well as of infectious cDNA clones with a duplicated 35S promoter of CaMV.

The genus Cucumovirus contains three virus species, cucumber mosaic virus (CMV), tomato aspermy virus (TAV) and peanut stunt virus (PSV), all economically important plant pathogens (Palukaitis et al., 1992). Member viruses of this genus have a tripartite single-stranded RNA genome of messenger sense, similar to the other three genera (Bromovirus, Alfamovirus and Ilarivirus) of the Bromoviridae (Murphy et al., 1995). We have recently reported that the cucumoviruses encode a small overlapping gene 2b in addition to the four major genes (1a, 2a, 3a and coat protein) common to the four genera (Ding et al., 1994; Shi et al., 1997). Analyses of the recently published sequence data suggested that the ilarviruses also encode a cucumoviral 2b-like gene (Ding et al., 1995b; unpublished results).

Mutational analyses have shown that the 2b gene of the Q strain of CMV encodes a host-specific long-distance virus movement function (Ding et al., 1995b). Recombinant assays showed that the 2b gene of V-TAV can functionally substitute for that of Q-CMV in at least six host species shared by the parental viruses (Ding et al., 1996) but not in cucumber (unpublished results), which is a systemic host for Q-CMV but immune to V-TAV (Habili & Francki, 1974).

To further study the host specificity controlled by different cucumovirus genes, it is essential to establish an efficient infection system for the cloned viral genomes of several representative species. Such a system is presently available only for strains of CMV, the type species of the genus (Hayes & Buck, 1990; Rizzo & Palukaitis, 1990; Suzuki et al., 1991; Boccard & Baulcombe, 1992; Zhang et al., 1994; Ding et al., 1995). We describe here the infectious full-length cDNA clones of TAV constructed in a modified pCass vector (pCass2) as well as in a pUC19-based pCass (pCass1). The original pCass vector was based on the plasmid pSP72 (Ding et al., 1995). We show that the infectivity of TAV cDNAs controlled under a partially duplicated CaMV 35S promoter in pCass2 is 3-fold higher than the same cDNAs driven by the single 35S promoter in pCass1.

Purification of V-TAV virions and viral RNAs was as described for CMV (Peden & Symons, 1973). The nucleotide (nt) sequences of the three genomic RNAs of V-TAV have been determined (Moriones et al., 1991; Bernal et al., 1991; F. García-Arenal, personal communication). First-strand cDNAs to the three V-TAV genomic RNAs were prepared as described previously for Q-CMV (Ding et al., 1995a). Second-strand synthesis followed the protocol in Sambrook et al. (1989) using RNaseH, Escherichia coli DNA polymerase I and E. coli DNA ligase, and cloning the resultant double-stranded DNAs into the Smal site of pBluescript SK(+). The near full-length cDNA clones selected for each of three RNAs, together with the missing 5′-terminal sequences obtained by PCR, were used for assembling the three full-length cDNA clones in pCass1 or pCass2 using a general strategy devised previously (Ding et al., 1995a). pCass1 was obtained by excising the 35S promoter...
and the 35S terminator from pCass and assembling them in the modified pUC19. The 35S promoter in pCass1 was partially duplicated from nt −410 to −90 as described (Kay et al., 1987) to yield pCass2 (Fig. 1a). This partial duplication resulted in at least a 10-fold increase in expression levels of foreign genes in transgenic plants (Kay et al., 1987). The three cDNA clones corresponding to TAV RNAs 1, 2 and 3 constructed in pCass1 were designated pCass1T1, pCass1T2 and pCass1T3 respectively and those in pCass2 were designated pCass2T1, pCass2T2 and pCass2T3, respectively (Fig. 1b).

The full-length cDNA clone of each of three genomic RNAs was purified by Superose 6 gel filtration chromatography (Skingle et al., 1990) and partially sequenced at the 5′- and 3′-terminal regions to confirm the integrity of the promoter fusions and identity to the published sequences. Several variations were observed in the 3′ untranslated regions of the cDNAs 1 and 3 as compared to the published sequences of the same strain of TAV (Bernal et al., 1991; F. García-Arenal, personal communication). The variations included one insertion (ATT between nt 3312 and 3313) in cDNA 1 and two substitutions (G→T and T→G) in cDNA 3 and one T insertion between nt 2369 and 2371 of cDNA 3. Importantly, the insert (ATT) in cDNA 1 creates a recognition site for EcoRI which was confirmed by digestion with this enzyme.

For assaying infectivity, each of the purified three genomic plasmid cDNAs was digested with a restriction endonuclease and the insert (ATT) in cDNA 1 creates a recognition site for EcoRI in which transcription starts is methylation sensitive and therefore pCass2 should be propagated in dcm− strains of E. coli, such as JM110. The insertion site between nt 2369 and 2371 of cDNA 3. Importantly, the insert (ATT) in cDNA 1 creates a recognition site for EcoRI which was confirmed by digestion with this enzyme.

For assaying infectivity, each of the purified three genomic plasmid cDNAs was digested with a restriction endonuclease and the insert (ATT) in cDNA 1 creates a recognition site for EcoRI which was confirmed by digestion with this enzyme.
Infectious full-length cDNA clones of TAV

Fig. 2. Viral RNAs in *N. glutinosa* plants inoculated with either cDNA clones or virions of V-TAV. (a) Systemic mosaic symptoms were induced with pCass2T1T2T3 (left top) or pCass1T1T2T3 (right top) or the wild-type virions (left bottom) or pCass2T1T2T3(Δ163) (right bottom) by manual inoculation. A control healthy *N. glutinosa* plant with mock-inoculation is shown in the middle on the right. (b) Electrophoresis analysis on a 1.2% agarose gel stained with ethidium bromide (EB) of encapsidated RNAs extracted from *N. glutinosa* inoculated with the wild-type V-TAV (lane 1), pCass2T1T2T3 (lane 2) or pCass1T1T2T3 (lane 3). (c) Northern blot analysis of encapsidated RNAs. Virion RNAs extracted from *N. glutinosa* inoculated with the wild-type V-TAV (lane 4), pCass2T1T2T3 (lane 5) or pCass1T1T2T3 (lane 6) were electrophoresed on a 1.2% agarose gel containing 1.1% formaldehyde, transferred onto a Hybond-N+ membrane (Amersham) and hybridized with a strand-specific RNA probe complementary to the 3’-terminal 128 nt of all three genomic RNAs. The positions of viral RNAs 1, 2, 3, 4, 4A, 3B and 5 are indicated, of which RNA 3B is a newly discovered subgenomic RNA derived from RNA 3 (Shi *et al.*, 1997b).
infected with pCass1T1T2T3 (the mixture of three genomic cDNA clones under the control of the single promoter) or pCass2T1T2T3 (the mixture of three genomic cDNA clones under the control of the partially duplicated promoter) as determined by RNA dideoxynucleotide sequencing (Ding et al., 1995) were all identical to each other as well as to those of the respective plasmid DNA inocula with one following exception. One U insertion was found between nt 2 and 3 of both progeny RNAs 1 and 2 derived from pCass2T1T2T3. This extra U has been persistently maintained in several subsequent passages and had no apparent effect on symptomology. It is not clear how this insertion occurred or whether it is related to the partial duplication of the CaMV 35S promoter. The relative infectivity of the V-TAV cDNA clones constructed in pCass1 and pCass2 was examined in two Nicotiana species, N. glutinosa and N. clevelandii. Each of the 13 dilutions from either set of the TAV cDNA clones was inoculated onto 30 seedlings (two replicates of 15 plants each) of either plant species and the percentages of plants infected were recorded 45 days after inoculation. The following points are evident from the data presented in Fig. 3. (i) When using lower amounts of plasmids as inocula the relative infectivity of the pCass2-based TAV cDNA clones was clearly higher (3-fold) than that of the pCass-based clones; however, the difference was not obvious for higher amounts of plasmid DNAs as inocula. It is most likely that the relatively higher infectivity of pCass2-based TAV clones resulted from a higher level of transient transcription as directed by the partially duplicated promoter inside the inoculated cells. It should be pointed out that our data do not exclude the extra U found in RNAs 1 and 2 derived from pCass2-based clones being the cause of higher infectivity. (ii) There is an obvious difference in susceptibility to virus infection from plasmid inocula between the two host species. For the same amounts, the percentage of N. clevelandii plants which became infected is always higher than that of N. glutinosa plants. (iii) Low amounts of plasmid DNAs (0.2 to 2 µg each of the three plasmids per plant) were sufficient to achieve an infection rate over 50%. Thus, a large-scale plasmid DNA preparation can generate a substantial amount of virus inoculum. Further infectivity assays using pCass2T1T2T3 demonstrated that the plasmid DNAs as inocula were as effective as viral particles on six additional host species: Chenopodium amaranticolor, Datura stramonium, Gomphrena globosa, Lycopersicon esculentum, Physalis floridana and N. tabacum cv. White Burley. However, up to 45 µg of pCass2T1T2T3 (45 µg each of pCass2T1, pCass2T2 and pCass2T3) failed to infect cucumber, which is a non-host of V-TAV (Habili & Francki, 1974). Thus, our data indicate that the host range of the plasmid inocula reflected the true host range of V-TAV when nine plant species were examined. We have recently characterized a novel subgenomic RNA 3B associated with V-TAV (Shi et al., 1997b). This RNA is 486 nt long and identical in sequence to the 3′-486 nt of RNA 3. RNA 3B consists of three characteristic regions, two tandem repeats of 163 nt each at the 5′ terminus and the 3′-terminal sequence of 160 nt which is highly conserved among all TAV RNAs characterized to date. A mutant of the RNA 3 cDNA clone (pCass2T3) was constructed by deleting one such repeat and was combined with the wild-type RNAs 1 and 2 cDNA clones to inoculate seedlings of N. glutinosa and N. clevelandii in addition to the above six host species. Northern blot analysis of RNAs from the inoculated plants showed the accumulation of all known V-TAV RNAs and the expected decrease in size of RNAs 3, 4 and 3B, thus confirming that RNA 3B was derived from RNA 3 (Shi et al., 1997b). However, no difference was observed between the wild-type and mutant viruses in symptomology (data not shown) and host range, suggesting a non-essential role for the additional copy of the tandem repeat that is absent in strains B, C and P of TAV (O’Reilly et al., 1991, 1994; Salanki et al., 1994). Efficient infection was achieved previously using the full-length cDNAs of CMV genomic RNAs cloned in a new plasmid vector, pCass (Ding et al., 1995a). We describe here a significantly improved version of pCass by partially duplicating the 35S promoter controlling the transcription of cloned
viral cDNAs. An increase in the relative infectivity was evident for the full-length TAV cDNAs cloned in pCass2 over the same cDNAs cloned in pCass1. These infectious cDNA clones of CMV (Ding et al., 1995a) or of TAV (in this study) have been used successfully for mutational analysis of the virus genome, constructing inter- or intra-species hybrid viruses, reconstituting pseudorecombintants between the two viruses and as the helper for satellite RNAs (Ding et al., 1995a, b, 1996; Shi et al., 1997b). Compared with other strategies currently used to obtain progeny virus from cDNA clones such as infectious RNAs transcribed in vitro from cDNA clones (Boyer & Haenni, 1994) and particle bombardment of cDNA clones (Gal-On et al., 1995; Fakhfakh et al., 1996), the pCass2 system we developed offers several unique features including easy preparation of plasmid inocula and plant infection by conventional mechanical inoculation, and thus is simple to use and cost-effective.

The authors wish to thank Fernando García-Arenal for kindly supplying us with the unpublished sequence of V-TAV and Wanxiang Li for technical help. This work was supported by the Australian Research Council Special Research Centre for Basic and Applied Plant Molecular Biology. B.-J.S. is the recipient of a University of Adelaide Overseas Postgraduate Research Scholarship.

References


Received 27 November 1996; Accepted 14 January 1997