Potato mop-top virus: the coat protein-encoding RNA and the gene for cysteine-rich protein are dispensable for systemic virus movement in Nicotiana benthamiana

Eugene I. Savenkov, Anna Germundsson, Andrey A. Zamyatin, Jr, Maria Sandgren and Jari P. T. Valkonen

1Department of Plant Biology, Swedish University of Agricultural Sciences (SLU), S-750 07 Uppsala, Sweden
2Department of Applied Biology, University of Helsinki, Finland

Full-length genomic cDNA clones of the Swedish isolate of Potato mop-top virus (PMTV) were transcribed in vitro using T7 RNA polymerase. The combination of RNA 1, 2 and 3 synthesized in the presence of m7GpppG cap analogue was infectious when inoculated onto Nicotiana benthamiana plants. Also, the combination of RNA 1 (encodes the viral replicase) with RNA 3 (encodes the triple gene block proteins and a small cysteine-rich protein (CP)) was infectious and both RNAs moved systemically in N. benthamiana plants in the absence of RNA 2, which encodes the coat protein (CP). However, the yellow mosaic symptoms that typically developed following PMTV infection with all three RNAs were not observed in plants infected with RNA 1 + RNA 3. Site-directed mutagenesis experiments revealed that expression of the putative CRP was not required for systemic infection and symptom induction in N. benthamiana. These data show that PMTV represents an example of a multipartite virus capable of establishing systemic infection without the CP-encoding RNA, and also without the putative CRP.

Potato mop-top virus (PMTV) is an economically important member of the genus Pomovirus and the causal agent of the serious potato tuber ‘spraying’ disease (Sandgren et al., 2002). Despite its economic importance (Sandgren et al., 2002) and the fact that it was described in the mid-1960s (Calvert & Harrison, 1966), PMTV has not been extensively studied due to the experimental limitations imposed by an RNA genome and the lack of infectious cDNA clones.

PMTV is the type member of the Pomovirus genus and is characterized by a tripartite, single-stranded RNA genome, the components of which are separately encapsidated within rod-shaped virions (Harrison & Jones, 1970). The nucleotide sequences of RNA 2 and RNA 3 of isolate T (Todd; Scottish) of PMTV (Scott et al., 1994; Kashiwazaki et al., 1995) and the complete nucleotide sequence of the Swedish isolate Sw of PMTV have been determined. The sizes of the RNA components of PMTV-Sw are 6043 kb (RNA 1), 3134 kb (RNA 2) and 2964 (RNA 3) (Savenkov et al., 1999; Sandgren et al., 2001; Savenkov, 2002). The 3'-terminal tRNA-like structure is identical in all three genome components (Savenkov et al., 1999). RNA 1 encodes polyproteins of 148 kDa and 206 kDa, which are thought to be components of the viral RNA-dependent RNA polymerase (RdRp), with the second protein probably being expressed by translational readthrough of the first cistron (Savenkov et al., 1999). RNA 2 encodes the coat protein (CP) and a readthrough protein (CP-RT) (Kashiwazaki et al., 1995; Sandgren et al., 2001); the latter is presumably involved in transmission by the vector Spongospora subterranea (Reavy et al., 1998). RNA 3 encodes the triple gene block (TGB) of movement proteins and a putative, small cysteine-rich 8 kDa protein of unknown function (Scott et al., 1994).

It has recently been shown that the genomic RNAs of PMTV in host plants constitute a heterogeneous population consisting of full-length copies and deletion variants of RNA 2 and RNA 3, regardless of whether the virus is propagated by mechanical inoculation or transmitted by the natural vector (Torrance et al., 1999; Sandgren et al., 2001). In order to produce a homogeneous inoculum for functional analysis of the PMTV genome and to permit manipulation of the PMTV genome, full-length PMTV cDNAs under a bacteriophage T7 RNA polymerase promoter were generated in this study.

PMTV-Sw was obtained from infested field soil using Nicotiana benthamiana as a bait plant. The total RNA was isolated from roots as described by Sandgren et al. (2001). RT-PCR using Pfu DNA polymerase (Stratagene) (an enzyme possessing a proofreading activity) and PMTV-specific primers were employed to amplify regions of the PMTV...
genome. The PCR products were digested with appropriate restriction enzymes, cloned, sequenced and joined together in pUC19 plasmids to produce full-length PMTV cDNAs (Fig. 1A). Two full-length cDNA clones for each genomic RNA were chosen for further experiments and all eight combinations of RNA 1, RNA 2 and RNA 3 were tested for infectivity on N. benthamiana.

In vitro transcripts were synthesized from MluI-linearized (RNA 1 and RNA 2) or SpeI-linearized (RNA 3) template DNA using bacteriophage T7 RNA polymerase (Promega) in the presence of 0.5 mM m'7GpppG (cap analogue) (New England Biolabs) and RNasin (Promega). Transcripts were analysed in 1% agarose gels, and an RNA size marker (Invitrogen) was used to estimate the size of the transcripts.

Of the eight combinations of RNA 1, RNA 2 and RNA 3 transcripts mechanically inoculated onto 3-week-old N. benthamiana seedlings, four induced yellow mosaic symptoms typical of PMTV infection in the upper non-inoculated leaves at 10 days post-infection (p.i.). The symptomatic leaves were positive for PMTV CP antigen as determined by DAS-ELISA at 14 days p.i. (data not shown). The non-infectious combinations all included transcript of the same cDNA of RNA 3, indicating that this particular clone was not infectious. Since both transcripts of the other two PMTV cDNAs were found to be infectious, only one cDNA clone of each RNA was used in further experiments. The infectivity of the in vitro transcripts was high on N. benthamiana (Table 1), and PMTV accumulated in the infected plants to titres similar to those observed in N. benthamiana plants infected by growth in soil infested with viruliferous S. subterranea (data not shown).

Studies on transgenic plants expressing PMTV CP have shown that the CP-encoding RNA of PMTV can be eliminated as a result of a resistance mechanism specifically targeted against RNA 2 (McGeachy & Barker, 2000; Germundsson et al., 2002). However, the loss of RNA 2 did not significantly affect accumulation and movement of RNA 1 and 3, suggesting that the CP-encoding RNA is dispensable for systemic virus movement. Mechanical inoculation of the virus from the systemically infected leaves of these transgenic plants to healthy, non-transgenic N. benthamiana plants resulted in systemic infection with RNA...
1 and RNA 3 (McGeachy & Barker, 2000). In our study, 3-week-old, non-transgenic N. benthamiana seedlings were inoculated with a mixture of in vitro transcripts of RNA 1 and RNA 3 cDNA. In contrast to N. benthamiana plants challenged with the inoculum containing all three genomic RNA components of PMTV, no symptoms were observed and no viral CP antigen was detected in plants inoculated with RNA 1 and RNA 3 transcripts. Nucleic acids were isolated from the upper non-inoculated leaves of at least six plants per type of inoculum and the samples were examined for accumulation of viral RNAs using RNA dot blot and Northern blot analysis. A probe for the tRNA-like structure identical in all PMTV genomic RNAs (Fig. 2A) and specific probes for RNA 1 and RNA 2 (Fig. 2B) were used. As expected, high levels of PMTV RNAs accumulated in the upper non-inoculated leaves of N. benthamiana plants challenged with the three-component inoculum (Table 1; Fig. 2). Also, inoculation with a combination of RNA 1 and RNA 3 resulted in systemic infection in five of nine inoculated plants (Table 1; Fig. 2), but the infected plants were symptomless. These results directly show that RNA 1 and RNA 3 can move systemically in the absence of RNA 2, although the infection of N. benthamiana with RNA 1 and RNA 3 appears to be somewhat less efficient than with the three genomic RNA components. Furthermore our results, along with the previous findings (McGeachy & Barker, 2000), indicate that a combination of the three genomic RNA components is required to produce a disease phenotype (yellow mosaic) characteristic of PMTV in N. benthamiana.

It has been shown recently that CRPs of peclu- (Dunoyer et al., 2002), tobra- (Liu et al., 2002) and hordeiviruses (Yelina et al., 2002) appear to be suppressors of RNA silencing and regulate the level of virus accumulation and systemic movement and influence symptom expression. Moreover, CRPs of hordei-, peclu-, furo- and tobraviruses share some degree of conservation (Savenkov et al., 1998). However, unlike these other virus genera, in which all species have an ORF for a CRP, in the genus Pomovirus only PMTV encodes a putative CRP. The putative 8K CRP of PMTV has no similarity to any known viral or cellular protein (Savenkov, 2002).

To address the possible role of the putative CRP in systemic virus movement and symptom expression, three mutant cDNA clones of RNA 3 were constructed (Fig. 1B). The

**Table 1. Infectivity on N. benthamiana of in vitro transcripts of PMTV and two mutants of PMTV RNA 3**

<table>
<thead>
<tr>
<th>RNA inoculum*</th>
<th>Infected/ inoculated†</th>
<th>Mean % infected</th>
<th>Symptoms‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>t1 + t2 + t3</td>
<td>13/14</td>
<td>93</td>
<td>YM</td>
</tr>
<tr>
<td>t1 + t3</td>
<td>5/9</td>
<td>56</td>
<td>NS</td>
</tr>
<tr>
<td>t1 + t2 + t3-CRP-stop</td>
<td>9/10</td>
<td>90</td>
<td>YM</td>
</tr>
<tr>
<td>t1 + t2 + t3-CRP-del</td>
<td>10/10</td>
<td>100</td>
<td>YM</td>
</tr>
<tr>
<td>t1 + t2 + t3-CRP-25aa-knock-out</td>
<td>6/6</td>
<td>100</td>
<td>YM</td>
</tr>
<tr>
<td>t1 + t3-CRP-stop</td>
<td>2/6</td>
<td>33</td>
<td>NS</td>
</tr>
<tr>
<td>t1 + t3-CRP-del</td>
<td>1/6</td>
<td>17</td>
<td>NS</td>
</tr>
<tr>
<td>t1 + t3-CRP-25aa-knock-out</td>
<td>–§</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Mock</td>
<td>0/6</td>
<td>0</td>
<td>NS</td>
</tr>
</tbody>
</table>

*5’-capped in vitro RNA transcripts (t).
†The number of plants as tested by ELISA and/or dot-blot hybridization; data are combined from two or three independent experiments. In ELISA, absorbance values (A405) threefold higher than those for non-inoculated control plants (A405 = 0.037–0.097) were considered as PMTV-infected.
‡YM, yellow mosaic; NS, no symptoms.
§–, Not tested.

**Fig. 2.** Accumulation of the PMTV genomic RNAs in the upper non-inoculated leaves of N. benthamiana 14 days p.i. assayed by Northern blot analysis. The combinations of RNA transcripts (t) used for inoculation are indicated at the top. (A) 32P-labelled desoxyriboprobe complementary to the tRNA-like structure was used. This probe detects all three PMTV genomic RNAs. sgRNA indicates a subgenomic RNA of the expected size predicted for expression of TGBp2 and TGBp3. (B) A mixture of two 32P-labelled desoxyriboprobes complementary to ~1600 5’-terminal nucleotides of RNA 1 and RNA 2 was used. These probes do not detect RNA 3 and sgRNA.
mutant pPMTV-3-CRP-stop was obtained by replacing the sequence between the unique MunI (nt 1991–1996) and SpeI (nt 2964–2969) sites with two equivalent PCR fragments into which the mutations had been introduced by overlap extension mutagenesis. Two PCR fragments digested with MunI/StuI and with StuI/SpeI were ligated into the MunI/SpeI-digested pPMTV-3. Four point mutations within the CRP ORF produced two in-frame stop codons and consequently, a C-terminally truncated CRP of 25 aa. The CRP ORF produced two in-frame stop codons and consequently, a C-terminally truncated CRP of 25 aa (2-9 kDa) (Fig. 1B). To construct pPMTV-3-del-CRP, in which the 3′-proximal part of the ORF for CRP was deleted, the MunI/StuI-digested PCR fragment and the HincII/SpeI fragment (nt 2667–2964) from pPMTV-3 were ligated into MunI/StuI-digested pPMTV-3. Mutant pPMTV-3-CRP-25aa-knock-out was made in order to eliminate the residual 25 N-terminal amino acids in the pPMTV-3-del-CRP mutant. It was obtained by replacing the sequence in the pPMTV-3-del-CRP between the unique MunI and SpeI sites with an equivalent PCR fragment and with the HincII/SpeI fragment from pPMTV-3. The PCR fragment was amplified using two nested primers, which mutate the CRP AUG and the beginning of the CRP ORF in such a way that the amino acid content of the overlapping 21K gene is not affected (the resulting non-synonymous mutations were as follows: MetVal, Argopal stop, PheSer, Isoleamber stop, ValPhe, HisTyr, Cys14Arg, Cys16Arg, Cys18Arg, Tyr19His, CysVal20Ala and Ser22Pro). The fidelity of all constructs was confirmed by sequencing.

RNA 1 and RNA 2 transcripts and each mutated RNA 3 transcript were inoculated onto 4-week-old N. benthamiana plants using three-component (RNA 1, 2 and 3) and two-component (RNA 1 and 3) transcript inocula. Most (90–100 %) of the plants inoculated with the three-component inoculum were infected and developed the typical symptoms of PMTV infection by 10 days p.i., demonstrating that CRP is not needed for PMTV symptom induction in N. benthamiana. On the other hand, as before, all plants inoculated with the two-component (RNA 1 + RNA 3) inoculum developed no symptoms. The non-inoculated upper leaves were harvested at 14 days p.i. and subjected to DAS-ELISA and dot-blot hybridization (Table 1). Most plants inoculated with the three-component inoculum were infected, consistent with symptom expression, but only a few of the plants inoculated with the two-component inoculum were infected, as before. Northern blot analysis of RNA extracted from the systemically infected leaves and using a probe specific for the PMTV tRNA-like structure revealed two major bands, of which one corresponded to the size of RNA 1 and the second one to RNA 2 and RNA 3, which are quite similar in size and coincide on a blot (Fig. 2A, lanes 4–6, and data not shown). A third small RNA, the size of which corresponded to a putative predicted subgenomic RNA 3, was detected as well. The subgenomic RNA was predicted on the basis of the mode of TGB expression in other viruses that utilize a subgenomic RNA to express TGBp2 and TGBp3 by leaky scanning (Morozov et al., 1991; Verchot et al., 1998). The bands corresponding to RNA 1 and RNA 3 were detected in RNA samples from symptomless systemically infected leaves in plants inoculated with the two-component inoculum (Fig. 2A, lanes 2 and 3). No RNA 2 was detected in the samples prepared from plants inoculated with the two-component inoculum (Fig. 2B, lanes 2 and 3).

Taken together, our results establish that the in vitro-generated RNA transcripts of full-length cDNAs for the three genomic RNAs of PMTV are highly infectious upon mechanical inoculation onto N. benthamiana plants, inducing symptoms and accumulating to titers similar to the original PMTV isolate. The combination of RNA 1 and RNA 3 transcripts was also infectious and able to establish systemic infection, which suggests that PMTV RNA 1 and RNA 3 are transported over long distance in plants in a non-virion form (McGeachy & Barker, 2000). Dispensability of CP for systemic virus movement has been reported for Barley stripe mosaic virus (BSMV), another tubular virus related to PMTV (Petty & Jackson, 1990). A ribonucleoprotein (RNP) consisting of the virus movement protein (TGBp1) and viral RNA has been purified and characterized (Brakke et al., 1988). It is thought to represent the transportable non-virion form of the virus. By analogy to the aforementioned viruses, one may speculate that PMTV spreads systemically as an RNP, probably a complex of TGBp1 and viral RNA. Tobacco rattle virus (TRV) is another well-characterized example of a tubular plant virus in which RNA 1 (RdRp-encoding RNA) is capable of replication and systemic movement in plants in the absence of the CP-encoding RNA (RNA 2) (Cadman, 1962; Harrison & Robinson, 1986), further showing that encapsidation and systemic movement can be uncoupled.

The second finding reported in this study is that CRP is not needed for the systemic spread of PMTV in N. benthamiana. In other well-characterized viruses CRP is typically not strictly essential for virus infection but is required for efficient systemic (long-distance) virus movement in planta and for symptom development, most likely as a consequence of the role of CRP in counteracting the host cytoplasmic RNA degradation mechanism (RNA silencing) targeted to viruses (Donald & Jackson, 1994; Dunoyer et al., 2001; Dunoyer et al., 2002; Liu et al., 2002; Yelina et al., 2002). Our data suggest that the CRP of PMTV is not required for such a purpose in N. benthamiana.

In conclusion, the data presented in this paper provide evidence that three genes, i.e. those for CP, CP-readthrough protein and CRP (or any sequence of RNA 2) are dispensable for systemic movement of PMTV in N. benthamiana. However, these findings do not exclude the possibility that these three genes might be necessary for compatible interactions in other PMTV hosts, a question which remains to be studied.

ACKNOWLEDGEMENTS

Financial support from The Royal Swedish Academy of Sciences (KVA), the Forestry and Agricultural Research Council (SJFR), grants
REFERENCES


