Research Note

The Coat Protein Is Dispensable for the Establishment of Systemic Infections by Pea Enation Mosaic Enamovirus

Jihad S. Skaf, Deborah G. Rucker, Steven A. Demler, Christiane E. Wobus, and Gustaaf A. de Zoeten

Department of Botany and Plant Pathology, Michigan State University, East Lansing 48824, U.S.A.

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Deletions in the coat protein (CP) open reading frame (ORF)-3 of pea enation mosaic enamovirus (PEMV) did not inhibit the systemic movement of PEMV. As early as 48 h postinoculation, truncated viral RNAs were detected in the roots and uninoculated leaves of infected plants. The detected concentrations of truncated RNAs varied significantly between experiments but were similar to those extracted from plants infected with wild-type (WT)-PEMV. Pea plants infected with CP-less mutants exhibited wilting and necrosis that appeared faster and were more severe than observed in infections with WT-PEMV. Moreover, the absence of the CP-ORF and its protein product did not affect the formation of either double-membrane bound vesicles or replication complexes characteristic of infections by PEMV. The potential role of these structures as the movement vehicles of the infectious entity of PEMV in plants is discussed.

Additional keywords: luteovirus, umbravirus.

The intricate interactions between the two single-stranded, messenger-sense, genomic RNAs of pea enation mosaic virus (PEMV) led to the classification of PEMV as the sole member of the genus *Enamovirus* (de Zoeten and Demler 1995). PEMV-RNA1 (5,706 nucleotides [nt]) displays notable similarities with the RNAs of subgroup II luteoviruses in gene arrangement and in the expression strategies of its genome (Habili and Symons 1989; Mayo et al. 1989; Demler and de Zoeten 1991; Koonin 1991; Koonin and Dolja 1993; Miller et al. 1995; Mayo and Ziegler-Graff 1996). RNA2 (4,253 nt) encodes a polymerase (Pol2) that is distinct from that encoded by RNA1. Pol2 is more closely related to the polymerases of carmo-like viruses encompassing subgroup I luteoviruses (Habili and Symons 1989; Koonin 1991; Demler et al. 1993; Koonin and Dolja 1993; Miller et al. 1995). The two PEMV RNAs have evolved to form a permanent partnership analogous to those described for the helper-dependent, aphid-transmitted virus complexes involving members of the genus *Luteovirus* and the recently established genus *Umbravirus* (Falk and Duffus 1981; Murrant 1993; de Zoeten and Demler 1995; Murrant et al. 1995; Demler et al. 1996). In the PEMV complex, the replicative competence of each RNA is contrasted by functional disabilities and interdependencies that are complemented in *trans* by the other RNA. The luteovirus-like component (RNA1), although the sole provider of both

structural and aphid transmission (AT) functions to the complex, is incapable of moving cell-to-cell or systemically via the phloem on its own, regardless of the inoculation method used (Demler et al. 1994, 1996). The umbravirus-like component (RNA2) provides functions essential for the movement of PEMV but lacks the genetic capabilities for its own encapsidation and AT (Demler et al. 1993, 1996). The ability of RNA1 to infect plants systemically is restored when coinoculated with the movement-competent RNA2. Plants infected by both PEMV RNAs 1 and 2 are characterized by relatively high concentrations of virions that can be detected readily in most cell and tissue types (Demler et al. 1996).

The inability of RNA1 to establish a systemic infection in the absence of RNA2, the mechanical transmissibility of PEMV (leading to a systemic invasion), and the loss of phloem limitation of PEMV infection are key divergences from all known luteoviruses (Miller et al. 1995; Mayo and Ziegler-Graff 1996; Demler et al. 1996). These unique features prompted this study of the role of the coat protein (CP) in the pathogenicity of PEMV.

Throughout this paper, we adopted the numbering used by Miller et al. (1995) for PEMVs open reading frames (ORFs) (Fig. 1). Partial or complete deletions in ORFs 3 and/or 5 were achieved by digesting the full-length, aphid-transmissible, transcriptionally active cDNA clone of WT-RNA1 (PEMV-AT⁺; Demler et al. 1997) with the various restriction enzymes shown in Figure 1. LCPAT0 lacks the 1,442-nt fragment extending from the *ScaI* (nucleotide 3934) to the *BstEII* (nucleotide 5376) sites. LCPAT1 lacks the 1,225-nt fragment from the *DraIII* (nucleotide 4151) to the *BstEII* sites. LCP3 lacks the *DraIII* to the *EcoRV* (nucleotide 4720) fragment (569 nt). LAT was constructed by removing the 502-nt fragment bracketed by two *ClaI* sites (nucleotides 4718 to 5220) (Fig. 1). Results shown in Table 1 demonstrated that transcripts from all of the RNA1 deletion mutants were infectious when coinoculated with RNA2 to pea (*Pisum sativum* L. '8221') plants. Most of the pea plants inoculated with transcripts from LCPAT0, LCPAT1, and LCP3 in conjunction with WT-RNA2 transcripts exhibited stunting, wilting, and acute necrosis (Fig. 2, and data not shown). These symptoms were more severe and appeared earlier (5 to 10 days postinoculation [dpi] for inocula containing RNA1 deletion mutants, compared with 7 to 10 dpi for symptoms produced by infection with WT-PEMV). In addition, infections with inocula containing RNA1 deletion mutants resulted in the death of the majority of inoculated pea plants. However, some plants as

well as shoots from lateral buds on symptomatic plants appeared symptomless. Both of the viral RNAs were readily detected in these symptomless plants and shoots by Northern (RNA) blot analysis (data not shown). To determine whether this severe symptom phenotype is specific to pea, *Nicotiana benthamiana* plants were also inoculated with the same RNA1 mutants (LCPAT0, LCPAT1, or LCP3) plus RNA2. The majority of uninoculated, newly formed leaves of *N. benthamiana* plants infected with the CP-deficient mutants (as determined by Northern blot hybridization analysis) remained symptomless. However, leaf curling and mild mosaic were occasionally observed on some uninoculated leaves of those plants (data not presented). These symptoms were much milder than those induced by infection with WT-PEMV. Pea and *N. benthamiana* plants infected with the RNA1 mutant LAT plus WT-RNA2 exhibited WT-PEMV-like symptoms (data not shown). The reason for this differential response of pea and *N. benthamiana* seedlings to infection by inocula containing the RNA1 mutants LCPAT0, LCPAT1, and LCP3 is not known. The construction of mutants with smaller deletions in the CP-ORF as well as the use of other test-plant species may help explain this phenomenon.

The only cytopathological difference associated with the marked alteration in symptoms induced by infection with the

RNA1 mutants lacking all or part of the CP-ORF was the absence of virions from nuclei, cytoplasm, or vacuoles of cells (data not shown). Interestingly, however, this absence of virions had no effect on the formation of replication complexes or the double-membrane bound vesicles characteristic of PEMV infection (de Zoeten et al. 1972; Burgess et al. 1974; de Zoeten et al. 1976; Powell and de Zoeten 1977; Powell et al. 1977; de Zoeten and Gaard 1983). It is well documented that RNA-dependent RNA polymerase activity, PEMV-specific dsRNA, as well as both viral ssRNAs are associated with these structures (Powell and de Zoeten 1977; Powell et al. 1977; G. A. de Zoeten, *unpublished data*). In addition, these vesicles, whose formation requires RNA1 (Demler et al. 1994), move throughout infected plants via the phloem (de Zoeten and Gaard 1983). These observations combined with the movement of CP-less RNA1 mutants reported in this paper further strengthen the hypothesis that PEMV moves throughout plants as a ribonucleoprotein complex. Thus, it is possible that the role of these vesicles in the movement of PEMV is to protect RNA1 (and possibly RNA2) from cellular ribonucleases. This is in contrast to other luteoviruses, in which the CP is thought to protect the genomic RNAs from ribonucleases (Reutenauer et al. 1993; Mohan et al. 1995).

Northern blot hybridization analysis of total RNA from

Fig. 1. Schematic representations of **A**, the genome structure of wild-type (WT) pea enation mosaic enamovirus (PEMV)-RNA1, and **B**, the various deletion mutants tested in this study. Hollow boxes are numbered according to Miller et al. (1995) and indicate open reading frames (ORFs). Calculated size of the various protein products is indicated in kDa. Restriction enzyme sites are according to the sequence of pPER1 published by Demler and de Zoeten (1991). Dashed lines indicate in-frame deletions. CP: coat protein, Pol: polymerase, AT: aphid transmission. Names of the mutant viruses are composed of "L" (for the luteovirus component) followed either by "CP" and/or "AT" (for the coat or aphid transmission proteins, respectively). LCPAT0 lacks the entire CP-ORF-3 and the 5'-87% of the AT-ORF5. LCPAT1 lacks 73% of the CP-ORF and the 5'-87% of the AT-ORF5. LCP3 lacks the same CP fragment as in LCPAT1 plus the 5'-157 nucleotide of the proline-rich region that immediately follows the CP-ORF (Demler and de Zoeten 1991). LAT has an intact CP-ORF but lacks the 5'-63% of its AT-ORF. Cleaved DNA fragments were separated on 1% agarose gels and corresponding restriction fragments were isolated with GeneClean II (Bio 101, Vista, CA). Incompatible sticky ends were filled in with the Klenow fragment of DNA polymerase I, and subsequently religated with T4 DNA ligase. Fidelity of ligations was confirmed by restriction fragment mapping and sequence analysis.

Table 1. Infectivity of transcripts from wild-type (pea enation mosaic enamovirus (WT-PEMV) and various RNA1 deletion mutants

Transcript combination ^a	Infected/total plants ^b
Mock	0/10
WT-RNA2 + WT-RNA1	19/20
WT-RNA2 alone	12/20
WT-RNA2 + LCPAT0-RNA1	17/20
LCPAT0-RNA1 alone	0/10
WT-RNA2 + LCPAT1-RNA1	15/20
LCPAT1-RNA1 alone	0/10
WT-RNA2 + LCP3-RNA1	15/20
LCP3-RNA1 alone	0/10
WT-RNA2 + LAT-RNA1	7/9
LAT-RNA1 alone	0/10

^a cDNA clones of WT-RNA2, WT-RNA1, and the various deletion mutants were linearized with *Sma*I, *Pst*I, and *Pst*I, respectively. Capped transcripts were synthesized with T7 RNA polymerase. Young, healthy pea and *Nicotiana benthamiana* plants were Carborundum-dusted and inoculated with 10 µg of the various RNA1 transcripts (alone or with WT-RNA2 transcripts) in 10 mM Tris-Cl (pH 8.0), 1.0 mM EDTA, 1% Bentonite. Plants were maintained in a growth chamber at 20°C.

^b Infectivity was assayed by monitoring symptom development and verified by Northern (RNA) blot hybridization analysis of total RNA from selected, systematically infected, symptomatic leaves (isolated 48 h to 6 weeks postinoculation) with RNA1- and RNA2-specific probes.

Fig. 2. Comparison of the symptoms induced by **A**, wild-type (WT) pea enation mosaic enamovirus (PEMV) 10 days postinoculation (dpi), and by **B**, the RNA1 deletion mutant LCPAT1 5 dpi. LCPAT1 lacks 73% of the coat protein open reading frame (ORF) and the first 87% of the aphid transmission ORF. Pea plants were inoculated with 10 µg of RNA1 transcripts (WT or mutant) along with WT-RNA2 transcripts and grown at 20°C.

uninoculated leaves was used to follow infection by PEMV. Since severely dwarfed pea plants infected with inocula containing RNA1 mutants LCPAT0, LCPAT1, and LCP3 often did not form new leaves, total RNA from the roots of infected plants also was analyzed. Both genomic and subgenomic RNAs of the RNA1 mutants with deletions in the CP and AT ORFs (LCPAT0, LCPAT1, and LCP3) were detected in the roots and in uninoculated leaves as early as 48 h postinoculation. This rapid movement of viral RNA, combined with the appearance of symptoms in upper, uninoculated leaves as early as 5 dpi, suggests vascular movement of the infectious entity. The differential mobility of genomic and subgenomic RNAs presented in Figure 3 indicates the absence of contamination by WT-PEMV-RNA1 and shows that infection is indeed caused by the RNA1 deletion mutants.

In Western blots (immunoblots), CP-specific bands were not observed when total protein extracted from pea plants infected with the various CP-deletion mutants was probed with antibodies against PEMV virions (data not shown). This confirms that CP (and hence virion formation) is not required for infection.

No consistent, significant difference in genomic or subgenomic RNA accumulation between WT and RNA1 deletion mutants was detected. The quantities of progeny RNA1 isolated from plants infected with RNA transcripts from the same construct (WT or mutant) varied widely between different experiments, plants, and tissue types (Fig. 3, compare lanes 5

Fig. 3. Northern (RNA) blot hybridization of total RNA extracted 5 days postinoculation (dpi) from uninoculated leaves (lanes 1 through 5) and roots (lanes 6 through 10) of pea plants inoculated with transcripts from WT-RNA1 + WT-RNA2 (lanes 1 and 6), WT-RNA2 alone (lanes 2 and 7), LCPAT0-RNA1 + WT-RNA2 (lanes 3 and 8), LCPAT1-RNA1 + WT-RNA2 (lanes 4 and 9), LCP3-RNA1 + WT-RNA2 (lanes 5 and 10). One microgram of RNA was separated through 1% agarose gel and hybridized against a digoxigenin (DIG)-11-UTP-labeled RNA probe that detects the sense-strand of RNA1 as described by Demler et al. (1994). The blots were scanned with the Nikon Coolscan plug-in with Adobe Photoshop (Adobe Systems, Mountain View, CA). The difference in the mobility of genomic (g) and subgenomic (sg) RNA reflects the extent of the deletion in the cDNA. The electrophoretic profile of RNA from the RNA1 mutant LAT is indistinguishable from that of the RNA1 mutant LCP3 (lanes 5 and 10) and is not shown in this figure.

and 10). Frequently, greater quantities of RNAs1 were obtained from similar amounts of tissues infected with truncated RNAs1, compared with WT-PEMV. This is in contrast to other luteoviruses, in which the accumulation of progeny RNA in infected protoplasts was greatly reduced (Reutenauer et al. 1993; Mohan et al. 1995). This makes a role for the CP of PEMV in stimulating genomic RNA synthesis unlikely. Such a possibility was not ruled out in the case of other luteoviruses (Reutenauer et al. 1993; Mohan et al. 1995). In contrast, the accumulation of RNA2 did not vary between different experiments or plants and was independent of the accumulation of WT- or truncated RNAs1 (data not shown).

Inocula containing the RNA1 mutant LAT, which lacks a significant portion (63%) of the AT-ORF, replicated and moved systemically similar to WT-PEMV and the other CP-deletion mutants. This was expected, since Demler et al. (1997) described a naturally occurring deletion mutant (AT Δ) lacking 727 nt in the 3' terminus of ORF5 of RNA1. AT Δ is mechanically (but not aphid) transmissible to whole plants when inoculated in combination with WT-PEMV-RNA2.

All of the RNA1 mutants could be passaged by mechanical inoculation, and the progeny viral RNAs appeared identical to the parental RNAs (as in Figure 3). However, none of these mutants could be transmitted to pea by aphids (*Myzus persicae* (Sulzer)) fed on plants infected with the RNA1 deletion mutants.

The CP-independent movement of PEMV reported in this paper is in sharp contrast to the absolutely necessary presence of the CPs for the movement of luteoviruses (Reutenauer et al. 1993; Mohan et al. 1995). Most viruses whose movement mechanism can be categorized as "tobamovirus-like" or "comovirus-like" require the CP and/or virion formation for vascular movement (Carrington et al. 1996; Séron and Haenni 1996), although there are exceptions (Petty and Jackson 1990; Scholthof et al. 1993). In contrast, umbraviruses are "naturally" CP-less viruses capable of establishing systemic infections in many plant species (Murant et al. 1995). Hence, RNA1 of PEMV is essentially a movement-defective luteovirus that has evolved to utilize the movement mechanism of a taxonomically unrelated, yet dependent, umbravirus.

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