Expression and suppression of circulative aphid transmission in pea enation mosaic virus

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Pea enation mosaic virus (PEMV) is composed of two autonomously replicating virus RNAs related to the genomic RNAs of viruses in the genera Luteovirus and Umbravirus. The transmission of PEMV resembles that of its luteovirus relatives in utilizing circulative aphid transmission. However, unlike its luteovirus counterparts, PEMV can also be mechanically transmitted. Prolonged mechanical passage of PEMV can lead to the loss of aphid transmissibility, a trait that is mirrored by specific changes in the PEMV virion composition. These changes were used to examine the virus contribution to vector transmission and the mechanisms by which it is regulated. Using a local lesion isolation technique, one aphid transmissible and two aphid non-transmissible isolates of PEMV were compared. Structural analysis of a 54 kDa minor structural subunit unique to the aphid transmissible isolate demonstrated that it was a fusion of the 21 kDa virus coat protein and a 33 kDa protein encoded immediately downstream of the 21 kDa ORF, consistent with the formation of the 54 kDa subunit by translational readthrough. Genetic analyses utilizing exchanges between infectious in vitro transcripts of each isolate demonstrated that although the 33 kDa protein was non-essential for infection, its presence was mandatory for aphid transmission, and that specific changes within the 33 kDa ORF were sufficient to confer or abolish aphid transmission. This study also demonstrates that isolates of PEMV exist as mixtures of aphid transmissible and non-transmissible genotypes, and provides insight into the mechanisms used by this virus to down-regulate aphid transmission in response to a specific selection pressure.

Introduction

Pea enation mosaic virus (PEMV), the type member of the genus Enamovirus, can be considered an irreversible mixed infection comparable in many respects to luteovirus helper-dependent complexes (Falk & Duffus, 1981; Murant, 1993). The bipartite genome consists of two autonomously replicating and taxonomically unrelated positive-sense RNAs. The larger RNA (RNA 1, 5706 nucleotides) possesses a strong, albeit imperfect, similarity in genomic organization and translated amino acid sequence to members of subgroup II luteoviruses (Demler & de Zoeten, 1991; Demler et al., 1994). In contrast, the smaller RNA (RNA 2, 4253 nucleotides) codes for a polymerase that is more closely aligned to a diverse group of viruses related to the genus Carmovirus (Demler et al., 1993, 1994). In this association, each RNA provides functions in trans that support apparent deficiencies in their counterpart. Although RNA 2 can autonomously infect whole plants, it is lacking a coat protein and is therefore dependent on the luteovirus component for structural and vector transmission functions. RNA 2 reciprocates by providing functions relating to systemic movement and mechanical transmission, functions lacking in RNA 1. Thus, although each RNA has some functional deficiencies, the RNA 1–RNA 2 combination is an effective infectious unit possessing properties unique to each of its individual components and their relatives.

It is the convergence and divergence between PEMV and its luteovirus counterparts that have made it an informative system for the study of luteovirus biology. One example of this concerns the vector and virus contribution to the circulative aphid transmission of each genus. In ultrastructural examinations of viruliferous aphids, parallels in the specific
association of virions with tissues of the digestive and salivary systems have been established (Harris & Bath, 1972; Harris et al., 1975; Harris, 1979; Demler et al., 1996; Gildow, 1987, 1990; Gildow & Gray, 1993). These observations have been interpreted as evidence of a receptor-mediated transport process. Similar parallels have also been demonstrated in the examination of the virus contribution to this process. The virions of both genera possess two structural proteins: a dominant coat protein of around 21–23 kDa with lesser amounts of a 50–74 kDa protein. Studies in luteoviruses have demonstrated that the large subunits are composed of the coat protein and a protein encoded immediately downstream of the coat protein ORF (Bahner et al., 1990; Vincent et al., 1991; Tacke et al., 1990; Dinesh-Kumar et al., 1992; Reutenauer et al., 1993; Cheng et al., 1994; Filichkin et al., 1994; Brault et al., 1995; Wang et al., 1995). Serological studies in PEMV have likewise demonstrated the presence of coat protein-specific epitopes in the large PEMV subunit, suggesting that it is also in part composed of the coat and a supplementary protein (Gabriel, 1983). Luteovirus in vitro expression studies have demonstrated that the single in-frame amber stop codon separating the coat protein gene from the downstream reading frame can be translationally suppressed. Hence readthrough translation has been postulated as a likely mechanism for the formation of this protein (Tacke et al., 1990; Dinesh-Kumar et al., 1992). In addition, the amino acid sequence of the coat and the N-terminal two-thirds of the putative readthrough proteins are strongly conserved between PEMV and luteoviruses, including the presence of a unique proline-rich region immediately following the coat protein gene stop codons. Combined, these similarities have led to the suggestion that the readthrough translation model developed for luteoviruses is also a likely explanation for the formation of the 54 kDa subunit in PEMV.

The role of these larger structural subunits in circulative aphid transmission is also based on complementary observations made in each genus. Isolates of PEMV that have been serially propagated solely by mechanical transmission will often have lost the capacity for aphid transmission, a characteristic that has been invariably correlated with the loss of this larger subunit (reviewed in Demler et al., 1996). Recent studies of natural variants of potato leafroll virus differentiated on their relative efficiency of aphid transmission demonstrated that regulation of this phenotype could be mapped to specific regions in the C terminus of the readthrough protein (Jolly & Mayo, 1994), although serological studies have also implicated the coat protein as containing determinants critical to the virus–vector interaction (van den Heuvel et al., 1993). Furthermore, mutational analyses of the beet western yellows virus (BWYV) genome (Brault et al., 1995) and that of barley yellow dwarf virus (Chay et al., 1996) have also demonstrated that changes within the readthrough domain were sufficient to interfere with or abolish aphid transmissibility. Thus, it has been suggested that these minor structural components of the PEMV and luteovirus virions represent proteins that are involved in aphid transmission.

Despite the similarities stated above, there are pronounced disparities between the PEMV and luteovirus proteins that suggest differences in both their function and mechanism of expression. One of these differences concerns the nucleotide context surrounding the coat protein gene stop codon. In luteoviruses, this region is strictly conserved (AAAUAGGUAGAC; Miller et al., 1995) whereas in PEMV this sequence is completely divergent (CUCUGAGGGGAC), including the substitution of an opal stop codon. In addition, the putative readthrough protein identified in luteoviruses (72–80 kDa) is significantly larger than the 54 kDa protein in PEMV. There is evidence that this larger luteovirus protein is processed into the 50–56 kDa version identified in purified luteovirus capsids (Bahner et al., 1990; Martin et al., 1990; Vincent et al., 1991; Cheng et al., 1994; Filichkin et al., 1994; Brault et al., 1995; Wang et al., 1995), though it is not clear whether this represents a mandatory maturation event or an experimental artifact. No such modification of the 54 kDa PEMV protein has been identified.

In the following study, we further dissect both the structure of and the necessity for the PEMV 54 kDa protein in aphid transmission. We demonstrate through structural analysis that the composition of this protein is consistent with the readthrough hypothesis developed in luteovirus systems. We furthermore demonstrate through genetic analyses that the aphid transmission of PEMV is directly linked to the presence of the 54 kDa subunit and also demonstrate two mechanisms in which the aphid transmission phenotype can be suppressed in response to the selection pressure imposed by mechanical transmission.

Methods

I Single lesion virus isolates. Three virus isolates were examined in this study, each of which was derived from an aphid transmissible Wisconsin isolate of PEMV (designated PEMV-NMT). This source isolate had been maintained under greenhouse conditions by serial aphid transmission at 3–4 week intervals in Pisum sativum. cv 8221 using the green peach aphid Myzus persicae (Sulzer).

Each isolate was purified from this aphid transmissible laboratory isolate through single lesion isolation. Twenty single lesion isolates were prepared by mechanical passage to the local lesion host Chenopodium quinoa. Well separated lesions were excised, ground in 0.1 M sodium acetate pH 6.0 containing 5% sucrose and inoculated onto individual pea seedlings dusted with carborundum. Following symptom development (7–10 days post-inoculation), 10 nymphs of M. persicae (Sulzer) were placed on each plant for a 2 day acquisition feeding period enclosed in a screen cage. The aphids were then transferred (five aphids per recipient plant) to recently emerged pea seedlings and allowed an inoculation access period of 2 days, at which point the aphids were asphyxiated. At 14 days post-inoculation, successful aphid transmission was assessed by symptom development and by northern blot analysis of total plant RNA (see below). Of 20 single lesion isolates examined, eight were positive for aphid transmission and one of these isolates was selected as the source material for isolate AT-.
Two aphid non-transmissible isolates were selected from the 12 aphid non-transmissible single lesion isolates derived above. Northern blot analysis of these local lesion isolates demonstrated that four of the 12 contained an RNA 1-specific RNA displaying an increased electrophoretic mobility relative to the RNA 1 derived from aphid transmissible lines (see below and Fig. 1). One of these four isolates was selected for further examination and was designated ATΔ. The other aphid non-transmissible isolate (designated AT+) was selected from the remaining eight aphid non-transmissible isolates that displayed RNA 1 mobilities identical to that of the AT+ isolate.

**Mechanically propagated isolates.** In addition to the three purified isolates described above, two additional PEMV laboratory isolates were also examined to illustrate the effect of repeated mechanical propagation of this virus. The aphid non-transmissible isolate used (PEMV-WSG) had been continuously propagated through serial mechanical propagation on pea seedlings for in excess of 10 years. The sequence and characteristics of this isolate have been reported previously (Demler & de Zoeten, 1991; Demler et al., 1993, 1994). In addition, a subculture of the aphid transmissible field isolate PEMV-NMT was also propagated for 20 successive passages by mechanical transmission onto pea seedlings.

**Virus purification and RNA analysis.** The procedures for large-scale virus purification and virus RNA isolation were as described previously (German & de Zoeten, 1975; Demler & de Zoeten, 1989). The production of total plant RNA, RNA 1-specific probe production and Northern blot analysis of non-denatured RNAs were also as described previously (Demler et al., 1994). Denaturing gel electrophoresis of 1–5 µg aliquots of virion RNA was performed using the glyoxal–formaldehyde denaturing procedure as described by Sambrook et al. (1989).

**Coat protein analysis.** The structural proteins of selected PEMV isolates were evaluated by electrophoresis of 1–2 µg aliquots of purified PEMV virions on SDS–polyacrylamide minigels (4% stacking gels, 12–15% separating gels) using the buffer system described by Laemmli (1970). The proteins were visualized by Coomassie blue staining.

Direct protein sequence analysis of intact and proteolytic fragments of the PEMV structural proteins was performed at the Michigan State University (MSU) Macromolecular Synthesis facility and at the University of Wisconsin-Madison (UW) Biotechnology Center. Aliquots (50 µg) of PEMV AT+ and AT− virions were suspended in 50 µl of Laemmli dissociation buffer and separated on SDS–polyacrylamide preparative gels (15% resolving gel, 4% stacking gel; Laemmli, 1970). The bands containing the 54 kDa and 21 kDa proteins were excised, the proteins electroeluted and concentrated by acetone precipitation. Aliquots (10 µg) of the purified PEMV AT+ 21 kDa and 54 kDa proteins and the PEMV AT− 21 kDa coat protein monomer were suspended in a buffer consisting of 20 mM phosphate pH 7.5, 150 mM NaCl and 10 mM EDTA and treated with 1–2 µg of the protease Igase (USB) for 16 h at 37 °C as described by the manufacturer. Approximately 5 to 10 µg aliquots of Igase-digested or undigested proteins were then suspended in 20 µl of Laemmli dissociation buffer and separated on SDS–polyacrylamide analytical minigels (15% resolving gel, 4% stacking gel; Laemmli, 1970). The proteins were then electrophoretically transferred to Immobilon-P membranes as described by the manufacturer (Millipore) using the CAPS–methanol transfer buffer described by Matsudaia (1987). The proteins of interest were detected by briefly staining the membrane (15 s) with 0.1% Coomassie blue in 40% methanol, followed by extensive destaining with 50% methanol. The blot was then air-dried and the bands of interest excised and subjected to direct N-terminal protein sequence analysis using a PE Applied Biosystem Model 494 Protein/Peptide Automated Sequencer (MSU) or an Applied Biosystems model 477A Liquid Phase Protein Sequencer (UW) following standard procedures.

**cDNA synthesis and cloning.** Numerical designation of primers and restriction sites are based on the previously reported sequence of PEMV RNA 1 (Demler & de Zoeten, 1991; GenBank accession number L04573). Complementary DNA copies of PEMV AT+, AT− and ATΔ were prepared using the AMV reverse transcriptase–T4 DNA polymerase procedure as outlined previously (Demler et al., 1993). The first strand primer 5’ dTTCAGCGATGATCGGATACAATTCCAG 3’ contains the complement of the 3’-terminal 15 nucleotides of PEMV RNA 1 (underlined), preceded by PstI and BamHI restriction sites. Second-strand synthesis with T4 DNA polymerase utilized the primer 5’ dTTCAGCGATGATCGGATACAATTCCAG 3’ which lies in the intergenic region preceding the RNA 1-encoded coat protein. The resultant double-stranded cDNA was then cleaved with PstI (3’ terminus) and NdeI (3746) and inserted into comparably cleaved pUC19. Successful recombinants were identified by restriction nuclease mapping and the sequence determined from both strands using the T7 DNA polymerase–dideoxynucleotide technique (Sequenase, USB; Tabor & Richardson, 1987).

**Construction of RNA 1 transcription vectors.** Full-length transcriptionally active RNA 1 clones coding for the coat protein and downstream regions of the AT+, AT− or ATΔ isolates were constructed by exchanging the Nedl–PstI fragment of each isolate into the previously described RNA 1 transcription vector pPER1 (Demler et al., 1994). In an effort to map the regions of RNA 1 critical to the regulation of aphid transmission, a series of reciprocal exchanges were created between the three vectors (see below). Restriction fragments for exchanges were isolated on 2% NuSieve GTG or 1% SeaPlaque agarose gels (FMC Bioproducts) and ligated into comparably digested vectors using standard procedures. The fidelity of all constructs was verified by sequence analysis. The protocol and vectors used for the production of capped RNA 1 and RNA 2 transcripts were identical to those described previously (Demler et al., 1994).
**Aphid transmission studies.** Evaluation of the aphid transmissibility of individual RNA 1 constructs was performed under growth chamber conditions in individual 4 inch pots. The seedlings were encased in a Magenta tissue culture box from which the bottom was removed and the top equipped with an interchangeable screened lid. Transcripts of individual RNA 1 constructs were co-inoculated with transcripts of RNA 2 onto pea seedlings (four to five seedlings per pot) as described previously (Demler et al., 1994). At 7 days post-inoculation, 10 to 15 non-viruliferous *M. persicae* nympha were transferred to individual symptomatic seedlings for an acquisition feeding period of 2 days. Successfully feeding aphids were then removed with a camel hair brush and transferred to recently emerged pea seedlings, with approximately five aphids per recipient seedling. Following a 2 day transmission feeding period, the aphids were asphyxiated and removed. The plants were then returned to the growth chamber and the success of aphid transmission was monitored by symptom development and Northern blot analysis. Each construct was evaluated in a minimum of three independent trials and each experiment included plants inoculated with the parental AT+ and AT− isolates as well as mock-inoculated controls.

**Results**

**Characterization of isolates: nucleic acid**

In previous studies of aphid transmission in PEMV, it was demonstrated that in response to repeated mechanical transmission, isolates of PEMV often developed into mixtures of aphid transmissible and non-transmissible genotypes. As this selection pressure was maintained, the aphid non-transmissible genotypes became the dominant species, eventually rendering the isolate aphid non-transmissible. The goal of this study was to examine this phenomenon in greater detail and correlate the emergence of this phenotype with specific characteristics of the virus capsid.

Through the use of the local lesion host *C. quinoa* we were able to demonstrate that a laboratory isolate of PEMV maintained solely by aphid transmission (PEMV-NMT) actually contained a variety of genotypes corresponding to both aphid transmissible and non-transmissible phenotypes. Of a total of 20 separate isolates initially characterized, eight were successfully transmitted by nymphs of *M. persicae*, whereas the remaining 12 were aphid non-transmissible. Although the emergence of the aphid non-transmissible phenotype under the selection pressure imparted by repeated mechanical propagation is well documented, this data clearly demonstrates that the co-existence of transmissible and non-transmissible genotypes also occurs under conditions in which propagation has been maintained solely by the insect vector.

Characterization by Northern blot analysis of total RNA derived from plants infected with these isolates demonstrated that two distinct types of aphid non-transmissible isolates had arisen (Fig. 1). As is evident in the blot, the isolate designated AT−Δ displays a reduction in the size of its RNA 1, as well as in the subgenomic RNA encoding the coat protein and 3′-terminal regions. In contrast, another aphid non-transmissible isolate (designated AT−) was of equivalent mobility to aphid transmissible isolates (AT+).

Denaturing gel electrophoresis of virion RNA derived from these isolates also verified this dichotomy (Fig. 2). Comparison of the virion RNA from the local lesion isolates AT+ and AT− clearly demonstrated an increased mobility of RNA 1 from the deletion isolate versus its full-length counterparts. Fig. 2 also confirms that the PEMV isolate containing the deletion variant RNA 1 is capable of forming stable virions. Indeed, the overall virus yield of the deletion variant was enhanced three to five times over that of the AT+ and AT− isolates containing full-length RNA 1 species. Although the progression of symptom formation was identical in all three isolates, the overall severity of the stunting, rugosity and the vividness of the yellow leaf mosaic symptoms caused by the AT−Δ isolate were more severe than those of the AT− and AT+ isolates.

**Analysis of structural proteins**

The structural proteins derived from purified virions of the AT+, AT− and AT−Δ isolates were examined by SDS–PAGE
and are depicted in Fig. 3(a). The particles of all three isolates contained the 21 kDa coat protein, and those of the AT⁺ isolate contained an additional minor component of 54 kDa. Although additional minor protein components of 44 kDa and 28 kDa have been reported associated with some isolates of PEMV (Hull, 1977a, b), we found no evidence of comparable species using the isolates and techniques described here.

Composition analysis of the 54 kDa subunit

To address whether the formation of the 54 kDa protein is consistent with the readthrough translation hypothesis, we examined the N-terminal amino acid sequence of both the intact and proteolytic fragments of the 54 kDa and 21 kDa proteins. N-terminal sequencing of the two purified coat proteins derived from the AT⁺ isolate and of the 21 kDa coat protein derived from the AT⁻ isolate gave the sequence MPTRSRS, which is consistent with the N-terminal sequence of the 21 kDa protein identified in previous sequence analysis (Fig. 3b; Demler & de Zoeten, 1991). These data also corroborate previous serological analysis of both PEMV and the luteoviruses, demonstrating that the larger 54 kDa subunit is in part composed of the PEMV coat protein (Gabriel, 1983).

Proteolytic mapping of the 54 kDa protein was performed with the endoprotease Igase, which recognizes the tetrapeptides PP/TP, PP/SP and PP/AP as well as pentapeptides in which the first proline has been replaced by proline–alanine (e.g. PAP/SP). If the formation of the 54 kDa protein involves readthrough translation, sequence data predicts that two cleavage sites corresponding to the tetrapeptide PPSP should be reflected in the product. Both recognition sites occur in the proline-rich region following the coat protein stop codon and should result in fragments of 22, 31 and 1 kDa (Fig. 3b; Fig. 4). Consistent with this hypothesis, digestion of purified 54 kDa protein with Igase resulted in two peptides of 22 and 31 kDa (Fig. 3b; the 1 kDa fragment was unresolved in this system). N-terminal amino acid sequencing of the 22 kDa peptide yielded the sequence MPTRSRS, identical to the N terminus of the coat protein. The N terminus of the 31 kDa peptide had the sequence SPTPVGA, which matches the second Igase cleavage site identified in the proline-rich region. Thus, both the digestion pattern and the sequence of the resulting fragments were consistent with the formation of the 54 kDa protein by readthrough fusion of the coat and 33 kDa ORF products.

Sequence comparison of the AT⁺ and AT⁻∆ isolates

Fig. 4 depicts the nucleotide and amino acid sequence generated from three individual cDNA clones derived from the AT⁺, AT⁻ and AT⁻∆ isolates. The region depicted corresponds to the 3’-terminal region, beginning in the intergenic region preceding the coat protein and extending to the 3’ terminus.

Although the virions of the AT⁺ isolate lacked the 54 kDa protein associated with aphid transmissible isolates, there was no obvious evidence in the nucleotide or amino acid sequence of a translational aberration that would lead to the omission of this protein. Both isolates encoded the full 21 kDa coat protein as well as the 33 kDa downstream ORF. A total of 37 nucleotide changes were identified in the analysed region, 10 of which occurred in the non-coding regions. Within the coding regions, 18 of the changes led to no change in the amino acid sequence, five led to conservative changes and the remaining changes led to three non-conservative amino acid substitutions. Although the coat protein stop codon context of PEMV is decidedly different from its luteovirus counterparts, the sequence of this region was identical between AT⁺ and AT⁻ isolates, with the exception of a single nucleotide alteration occurring 19 nucleotides upstream of the stop codon.

Sequence comparison of the AT⁻ and AT⁻∆ isolates

Through sequence analysis, the deletion evident in RNA 1 of the AT⁻∆ isolate can be mapped to the omission of a 727 nucleotide region beginning in the proline-rich coding region following the coat protein stop codon (nucleotide 4614) and extending to nucleotide 5342 (Fig. 4). At this point, the sequence shifts out of frame and extends for an additional 27 amino acids before termination.

A total of 38 nucleotide changes were evident in comparisons of the regions common to AT⁻∆ and AT⁺ isolates. Thirteen of these changes occurred in non-coding regions and an additional eight occurred in the coding region downstream of the coat stop codon. Within the coat protein-encoding region there were 17 changes, 14 of which led to no change in the amino acid sequence, two led to conservative substitutions and one led to a non-conservative substitution. As was the case with the full-length AT⁺ and AT⁻ isolates, the region surrounding the coat protein stop codon was identical in this isolate.

Although this deletion in the 33 kDa AT⁻∆ isolate would clearly account for the absence of the 54 kDa readthrough product, it should be noted that there was no evidence of a 26 kDa readthrough product, which would be anticipated if readthrough also occurred in this isolate. This suggests that either readthrough does not occur, that the truncated product is unstable, or that it is incapable of insertion into PEMV virions. This result is consistent with that reported by Chay et al. (1996) in which a synthetic truncation of a luteovirus readthrough protein was also undetectable in infected protoplasts. It should also be noted that in light of the competitive advantage displayed by this isolate, these data clearly demonstrate that the intact 33 kDa ORF is not essential for the virus.

Mapping of regions critical to aphid transmission

Using transcription vectors containing the coat and readthrough regions of the AT⁺, AT⁻ and AT⁻∆ isolates, a
To define further the change(s) in the 33 kDa ORF responsible for the regulation of aphid transmission, an additional series of hybrids was created utilizing restriction sites bordering internal nucleotide changes between the AT+ and AT− isolates (Figs 5 and 6). While the substitution of a 746 nucleotide fragment bordered by Smal sites was sufficient to reverse the aphid transmissibility of the parental isolates, a slightly smaller 500 nucleotide fragment defined by two ClaI sites was incapable of altering this phenotype. These exchanges suggested that the critical region controlling aphid transmission lies in the 131 nucleotide segment between the latter ClaI and Smal sites, a result confirmed in the final exchange defined in Fig. 5. Within this region were a total of five nucleotide replacements, three of which led to amino acid substitutions. Of the additional exchanges assembled within this region (Fig. 6), only those constructs containing the single A to G substitution at position 5282 (resulting in the replacement of an asparagine with serine) were capable of reversing the aphid transmissibility of the recipient isolate. This result was most clearly evident in the exchange defined by the Tth1111I–BamHI restriction sites, which contained only this single nucleotide substitution, and which also resulted in the reversal of the aphid transmissibility of the parental isolates. These data provide added support to the results described in the preceding paragraph linking the regulation of aphid transmission to specific changes within the 33 kDa ORF.

Although the parental AT− isolate used in these exchanges lacked the 54 kDa protein when analysed by SDS–PAGE, this final construct introducing the Tth1111I–BamHI fragment from the AT− source isolate into the AT+ background (resulting in a AT+ phenotype) did express the 54 kDa protein at levels comparable to the source AT+ isolate (data not shown). Although previous reports examining aphid non-transmissible PEMV isolates have invariably correlated the presence of the 54 kDa protein with an AT+ phenotype, this observation suggests that a dysfunctional 54 kDa protein may represent yet another mechanism through which aphid transmission is down-regulated. Owing to the large number of exchanges examined in this study, it has not been feasible at this time to assay all of the exchanges and their various permutations to identify to what extent the virus genome could be modified without preventing the appearance of 54 kDa protein in virus particles.
Discussion

Previous studies of the individual components of the PEMV genome have established strong taxonomic and biological links to members of the genera *Luteovirus* and *Umbravirus*, and to the helper-dependent complexes composed of these genera. The hybrid nature of the PEMV genome imparts to the virus attributes not evident in its luteovirus relatives; the dual capacity for aphid and mechanical transmission, the ability to establish a true systemic infection and a significant enhancement in virus titres. It was the goal of this study to exploit these traits to examine the interaction between

Fig. 4. For legend see page 519.
virus and vector in circulative aphid transmission, as well as to gain some insight of the response of a virus to a specific selection pressure (mechanical transmission).

Amino acid sequencing of the intact and proteolytically cleaved structural subunits of the PEMV capsid demonstrated that the 54 kDa protein associated with aphid transmissible isolates of PEMV is composed of a fusion of the coat protein and a downstream, in-frame ORF separated by a single opal
termination codon. This data augments previous serological and translational studies of luteoviruses in demonstrating the presence of peptides specific to both the coat protein and the proline-rich region of the 33 kDa ORF in this minor subunit, observations that support their formation by translational readthrough. Despite this similarity to luteoviruses, there are two distinct differences between these genera regarding the formation of this subunit. Unlike the luteoviruses, there is no evidence in PEMV of processing of the readthrough protein prior to or during virion maturation. The 54 kDa ORF product appears to be inserted into the capsid intact, with no evidence of the maturation or processing step hypothesized in the luteoviruses (Bahner et al., 1990; Martin et al., 1990; Vincent et al., 1991; Cheng et al., 1994; Filichkin et al., 1994; Braught et al., 1995; Wang et al., 1995). PEMV has apparently dispensed with the function of this appendage by eliminating this segment of the readthrough protein entirely from the viral RNA. The second notable difference concerns the difference in the coat protein termination codon context in PEMV versus its luteovirus counterparts. Although we have previously speculated that this difference may have been an explanation for the suppression of aphid transmission in this virus, the data in this study clearly demonstrates that this context is identical in both aphid transmissible and non-transmissible isolates. Therefore, either PEMV has evolved an entirely different set of signals to control the readthrough process, or, if this regulation is common in both genera, it occurs at a site remote from the stop codon itself.

A second goal of this study was to substantiate the circumstantial link between the 54 kDa protein and aphid transmission. Three local lesion isolates, differentiated on their support of aphid transmission, served as the source for cDNA clones covering the coat protein and readthrough regions. These clones, and heterologous exchanges between them, were then used for the production of infectious RNA transcripts to map regions critical to the expression of aphid transmissibility. Using this approach, we were able to demonstrate that the 33 kDa ORF is mandatory for the support of aphid transmission. Deletion of this region, either naturally as in the case of the AT−Δ isolate or in vitro, is sufficient to suppress this phenotype. In the isolates examined in this study, the reintroduction of this ORF from an aphid transmissible
Fig. 5. Mapping of the region of PEMV RNA 1 critical for the support of aphid transmission. Full-length cDNA clones differing only in the region from nucleotides 3746 to the 3’ terminus (encompassing the coat and 33 kDa ORFs) were assembled from isolates AT−, AT−Δ and AT+. RNA transcripts from the parental clones and from a variety of reciprocal exchanges were co-inoculated with transcripts of RNA 2 and the aphid transmissibility of each construct evaluated to map the regions of RNA 1 critical to the regulation of aphid transmission. Designations ‘+’ and ‘−’ to the right of each construct indicate support or abolishment of aphid transmission. These results demonstrate that the presence of the 33 kDa ORF is mandatory for the support of aphid transmission and that specific changes within this region are sufficient to both support or abolish vector transmission.

Fig. 6. Analysis of the region encompassing nucleotides 5200–5400 of PEMV isolates AT+ and AT− for the regulation of aphid transmission. The ‘+’ and ‘−’ designations to the right of each exchange again designate the support or lack of support of aphid transmission. Note that the single A to G nucleotide replacement at position 5282 (leading to a N to S amino acid replacement) is sufficient to regulate aphid transmission.
isolate was sufficient to reinstate aphid transmissibility, regardless of the source of the coat protein and non-coding regions. Supporting the observed necessity for the 33 kDa ORF, we were also able to demonstrate that progressively smaller exchanges within the 33 kDa ORF between the full-length AT- and AT+ isolates were sufficient to confer or abolish aphid transmission. Combined, these data provide strong support for the conclusions drawn by Jolly & Mayo (1996) linking the luteovirus readthrough proteins with aphid transmission. It is again important to state that this data does not preclude the virus coat protein or non-coding regions of PEMV RNA 1 from also playing a role in aphid transmission, only that among the limited number of isolates examined in this study, such a role for these regions was not uncovered (see e.g. van den Heuvel et al., 1993).

We were also able in this study to gain some insight into the mechanisms utilized by a virus in the down-regulation of a phenotype in response to a selection pressure. The emergence of aphid non-transmissible phenotypes in response to repeated mechanical transmission has been well established in PEMV and, in this case, two distinct mechanisms of suppression of aphid transmission were identified. In one case, the AT-Δ isolate, suppression was attained through the deletion of a substantial portion of the 33 kDa ORF, the occurrence of which may be favoured through specific features of the virus RNA sequence flanking the deletion site. The 5'-most region of the deletion lies within the proline-rich coding region following the coat protein stop codon, while the 3'-terminal segment lies in a smaller proline-rich coding region nearer the C-terminus (Fig. 4). The fusion point occurs immediately following a segment of seven consecutive C residues followed by seven consecutive G residues. Since prolines are encoded by CCN triplets, both of these regions are abundant in C nucleotides. Current models of virus RNA recombination rely on a multistep process (see e.g. Lai, 1992), initiated by the interruption of replication by RNA secondary structure. The replication complex then detaches from its template (with the nascent strand attached) and localized base pairing repositions the complex prior to the resumption of replication. The sequence of the AT-Δ isolate possesses the two elements necessary for such a scenario; the 3'-terminal C–G hairpin to interrupt replication and the two C-rich regions capable of facilitating limited base pairing to reposition the complex. These data also lend support to the phyletogenetic analysis of Gibbs & Cooper (1995) who identified the same two regions flanking the intramolecular deletion of the AT-Δ isolate as potential locations for intermolecular exchanges among PEMV and the luteoviruses during virus evolution.

The occurrence of this deletion isolate is also consistent with previous observations of this phenomenon reported by Adam et al. (1979) and provides clarification of conflicting reports of the size of RNA 1 cited by numerous authors (see Demler et al., 1996). This isolate was also notable in the three- to fivefold enhancement in virus titres over the AT+ and AT- isolates. This attribute may be a reflection of a more efficient replication of a shorter RNA or may reflect a greater stability of virions lacking a mixture of subunits. The dispensability of the PEMV 33 kDa ORF is in sharp contrast to the conclusions drawn by Brault et al. (1995) and Chay et al. (1996) who demonstrated that mutations in the readthrough domain of BWVV had a negative effect on virus accumulation as well as their demonstrated role in aphid transmissibility. This difference may represent another example of the complementation of functions provided in trans by RNA 2 to the PEMV RNA 1–RNA 2 interaction.

Like AT-Δ, the other suppressed isolate, AT-, also lacked the 54 kDa subunit and was aphid non-transmissible, yet was identical to the aphid transmissible isolate AT+ in the size of its RNA 1, its induced symptomatology and in the relative concentration of the virus in plants. At this time, there is no clear evidence on the mechanisms of suppression involved with this isolate. Although the lack of the 54 kDa subunit in the AT- isolate could be a result of down-regulation of the readthrough process, it is also possible that readthrough is in fact still occurring and that the product is incapable of efficient insertion into the capsid. Complicating this hypothesis was the observation that one artificial hybrid between AT- and AT+ isolates (defined by a single N to S amino acid substitution at position 5282) led to the presence of a 54 kDa protein in the resulting virions yet failed to support aphid transmission. It is therefore quite plausible that the down-regulation of aphid transmission in this AT- isolate may be occurring by more than one mechanism. It will remain for expansion of the studies described in this paper along with such approaches as in vitro translation and reporter gene expression studies to differentiate among these and other alternative mechanisms of regulation.

The data in this study also provide a vivid example of the quasispecies nature of a virus genome and the added flexibility this provides the virus in response to the selection pressures of its environment. In this and previous studies, it has been demonstrated that aphid transmissible isolates of PEMV consist of mixtures of aphid transmissible and non-transmissible species and that under the influence of a given selection pressure (mechanical transmission), aphid non-transmissible species become the dominant species. It is apparent from this flow towards aphid non-transmissibility that aphid transmission presents a negative selective pressure to this virus. In the case of the AT-Δ isolate, there was clearly a selective advantage over the aphid transmissible isolate as demonstrated by its enhanced virus titre. In the case of the AT- isolate used in this study, a comparable advantage was not evident, although others have reported that aphid non-transmissible isolates generally exceed their aphid transmissible counterparts in virus yield (French et al., 1973; Hull, 1977 b; Demler et al., 1996). Thus, the equilibrium distribution between AT- and AT+ genotypes would rest on the balance among a myriad of competing selection forces such as the necessity for
aphid transmissibility, the enhanced competitiveness of AT− genotypes, the enhanced transmission efficiency of higher titre isolates and so on. The cohesive force stabilizing this quasispecies is the ability of the PEMV coat and aphid transmission proteins to encapsidate RNA molecules of differing sizes and relationships to the full-length RNA 1 species. This reduced stringency of encapsidation permits the maintenance of AT− variants indefinitely, positioning the virus population for a rapid response to mechanical transmission. What is not clear at this time is whether the fluidity seen within the laboratory isolates used in this study is also mirrored in natural PEMV populations and whether the presence of aphid non-transmissible genotypes provides a comparable advantage to field isolates of this virus.

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References


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