Mutational evidence that the VPg is involved in the replication and not the movement of Pea enation mosaic virus-1

Jihad S. Skaf, Marilou H. Schultz, Hisae Hirata and Gustaaf A. de Zoeten

Department of Botany and Plant Pathology, Michigan State University, East Lansing, MI 48824, USA

Pea enation mosaic disease is caused by an obligatory association between the enamovirus Pea enation mosaic virus-1 (PEMV-1) and the umbravirus Pea enation mosaic virus-2 (PEMV-2). Encapsidated RNAs 1 and 2 are covalently linked to a 3138 Da VPg encoded by the RNA of PEMV-1. To determine the role of the VPg in the pathogenicity of PEMV (PEMV-1+PEMV-2), the infectivity of clones with mutations in key amino acids in the VPg was evaluated in protoplasts and in plants. Using quantitative, real-time RT–PCR, we concluded that the inability of certain mutants to infect plants was due to their replicative (and not their movement) incompetence. Mutant clones that produced delayed and less severe infections accumulated 10- to 100-fold less RNA-1 compared to WT-RNA-1 both in plants and in protoplasts. The RNAs of clones that produced WT-like infections accumulated to levels similar to those of WT-PEMV. Also, we demonstrate that the severity of symptoms produced by WT-PEMV is proportional to the amount of RNA-1 that accumulates in infected plants and seems to be independent of the amount of RNA-2. A dual role for the VPg in the pathogenicity of PEMV is proposed.

Introduction

The family Luteoviridae contains three genera, Enamovirus, Luteovirus and Polerovirus (d’Arcy et al., 1999). The genus Enamovirus currently contains a single virus, Pea enation mosaic virus-1 (PEMV-1). PEMV-1 participates in a disease complex with the umbravirus PEMV-2 to cause pea enation mosaic disease (Demler et al., 1996). In this complex, the replicative independence of the ssRNAs of both viruses is contrasted with the dependence of PEMV-2 on PEMV-1 for encapsidation and aphid transmission and by the dependence of PEMV-1 on PEMV-2 for mechanical inoculation and systemic movement (Demler et al., 1996; Skaf et al., 1999). In this paper, we use ‘PEMV’ to refer to the aphid-transmissible complex composed of PEMV-1 and PEMV-2.

One of the many strong, yet imperfect, similarities between PEMV-1 and poleroviruses is the presence of covalently linked viral genomic proteins (VPgs) at the 5’ termini of viral RNAs (van der Wilk et al., 1997; Wobus et al., 1998). VPgs have not been found in viruses belonging to the genus Luteovirus (Shams-Baksh & Symons, 1997).

VPgs are small proteins that are covalently linked to the 5’ termini of many viral genomes. Their function(s) may include:

- Priming RNA synthesis, signalling RNA encapsidation, and mediating RNA translation and protein cleavage (Nomoto et al., 1977a, b; Kuhn et al., 1988; Vartapetian & Bogdanov, 1987; Wimmer, 1982; Wittmann et al., 1997; Xiang et al., 1997).
- In the case of the potyvirus Tobacco etch virus, the VPg domain governs at least three functions: initiation of RNA synthesis, subcellular transport and host-specific long-distance movement (Schaad et al., 1997).

Using 125I labelling and protein electrophoresis, Reisman & de Zoeten (1982) documented the presence of a VPg covalently linked to the 5’ termini of viral genomic RNAs isolated from purified PEMV. More recently, using protein microsequencing, electrospray mass spectroscopy and total amino acid composition analysis, it was determined that the VPg of PEMV is composed of 28 amino acids (Wobus et al., 1998). The 3138 Da VPg is encoded by nucleotides 1811–1894 within ORF1 of RNA-1 downstream from the protease motif (Fig. 1). Sequence comparisons did not reveal any significant amino acid similarity between the VPg of PEMV and any other protein, including the VPg of the polerovirus Potato leafroll virus (Wobus et al., 1998). The context of the N- and C-terminal residues as well as the position and size of the VPg suggest that the mature VPg is generated via post-translational proteolytic processing of a polyprotein (encoded by ORFs 1 and 2 of RNA-1) which includes a membrane anchor, proteinase, the VPg and a polymerase (Wobus et al., 1998).
This study was undertaken to identify key amino acids in the VPg sequence that are required for efficient VPg activity and to determine the role of the VPg in the infection cycle of PEMV. In this paper, we demonstrate that mutations in key amino acids result either in abolition of the ability of PEMV to infect plants or in reduction in its infectivity, producing delayed and milder symptoms. Furthermore, we provide evidence that the reduced or abolished infectivity of certain VPg mutants can be attributed to reduced or abolished accumulation of RNA-1 as a result of suboptimal VPg sequence. These findings rule out a role of the VPg in PEMV movement.

Methods

- **Generation of VPg mutants.** The MORPH Site-Specific Plasmid DNA Mutagenesis Kit (5 Prime → 3 Prime, Inc.) was used to construct mutant clones of the full-length, infectious cDNA clone of the aphid-transmissible isolate (AT⁺) of PEMV-1 (pPER1, GenBank accession no. Y09099; Demler et al., 1994). Mutagenesis primers were chemically phosphorylated at their 5' termini, were composed of 28–33 nucleotides and encoded either one or two amino acid substitutions or deletions. Names of mutant clones are composed of the single-letter code and the position for the amino acid residue within or outside the VPg followed by the mutant residue. For example, Y2W is a mutant in which the tyrosine occupying the second amino acid position in the WT-VPg was changed to tryptophan; ∆ET is a mutant in which the putative 3C-like protease cutting site at the N terminus of the VPg was deleted; and Tfl4L refers to the mutant in which the threonine found outside the VPg (at position 4 in relation to the first amino acid) was replaced by a leucine. Also, a replicatively inactive clone (pBstEII) was generated by digesting pPER1 with BstEII (cuts at 5376) and was used as a negative control. After confirming the mutations by sequencing (Sequenase 2.0, Amersham; Tabor & Richardson, 1987), m7GpppG-capped transcripts from WT-PEMV-2 and WT-PEMV-1 or transcripts of PEMV-1 with mutated VPgs were synthesized in vitro by using T7 polymerase (Promega) as described previously (Demler et al., 1994).

- **Infectivity assays.** The infectivity of the various PEMV-1 clones was evaluated by observing the timing and severity of symptoms in pea
Involvement of VPg in replication of PEMV-1

(Phaseolus sativus L. cv. 8221). Pea seedlings (n \( \geq 40 \) plants per mutant) were inoculated with 5–10 \( \mu \)g of combinations of synthetic RNA transcripts of WT-PEMV-2 and WT-PEMV-1 or transcripts of PEMV-1 with mutated VPgs according to Demler et al. (1993). Total RNA was extracted from 100 mg newly formed leaves of both symptomatic and asymptomatic plants using the protocol described by Demler et al. (1994), or the SV Total RNA Isolation System (Promega). The isolated RNA was analysed by Northern blotting and/or RT–PCR as described below.

Pea protoplast assays. Pea protoplasts were isolated using a modification of the methods of Loesch-Fries & Hall (1980), Demler & de Zoenen (1989) and Demler et al. (1993). Briefly, 5 g portions of fully expanded leaves were collected from dark-conditioned, 2–4-week-old pea seedlings. The leaves were surface sterilized as described by Demler et al. (1993), and sliced with a sterile razor blade into 1–3 mm strips. The strips were preplasmolyzed by gentle shaking in 100 ml 10% mannitol for 1 h at room temperature. The strips were then digested in a 50 ml solution of 20 mg/ml Cellulysin (Calbiochem), 3 mg/ml Macerozyme R-10 (Calbiochem), 1 mg/ml BSA in 10% mannitol, pH 5.7. The tissue was digested for 2–5 h at 28 °C in a water bath/rotary shaker at ca. 100 rotations/min. The digest was filtered through three layers of sterilized cheesecloth and protoplasts were pelleted in a JA-20 rotor (that had been preplasmolysed by gentle shaking in 100 ml 10% mannitol, pH 5.7) and counted. Protoplasts were inoculated using the Perfectly Blunt Cloning Kit (Novagen). Cloned DNA was then sequenced to determine whether amplified sequences were cloned using the Perfectly Blunt Cloning Kit. PCR-products were analysed by agarose gel electrophoresis. Also, amplified RNA transcripts of WT-PEMV-2 and WT-PEMV-1 were translated in Rabbit Reticulocyte Lysate, Nuclease Treated System (Promega) according to the manufacturer’s instructions. Translation products were labelled with \( \mathrm{S}^{35} \)methionine for 60 min at 30 °C. The proteins were denatured by boiling the reactions for 2 min. Aliquots (10 \( \mu \)l) were loaded onto an SDS–polyacrylamide gel (Laemmli, 1970). Gels were dried and exposed on X-OMAT AR films (Kodak) for 16 h at room temperature.

Northern blot analysis. Approximately 1 \( \mu \)g of total plant or protoplast RNA was separated by electrophoresis in 1% nondenaturing agarose gels, blotted, hybridized to DIG-11-UTP-labelled RNA probes, and detected using the Genius System with Lumi-Phos 530 (Roche) as described by Demler et al. (1994).

In vitro translation. RNA transcripts (2 \( \mu \)g per reaction) of WT-PEMV-2 as well as WT- and mutant-PEMV-1 were translated in Rabbit Reticulocyte Lysate, Nuclease Treated System (Promega) according to the manufacturer’s instructions. Translation products were labelled in 50 \( \mu \)l reactions with translational-grade \( \mathrm{S}^{35} \)methionine for 60 min at 30 °C. The proteins were denatured by boiling the reactions for 2 min. Aliquots (10 \( \mu \)l) were loaded onto an SDS–polyacrylamide gel (Laemmli, 1970). Gels were dried and exposed on X-OMAT AR films (Kodak) for 16 h at room temperature.

RT–PCR. Primers flanking the VPg coding sequence (Fig. 1) were used in an RT–PCR protocol (Titan RT–PCR System, Roche) to amplify total RNA (1 \( \mu \)g) from plants and protoplasts. The forward primer DZ-82 (5′ CGGAATAGAAATACCTCTCTTAC 3′) was used with one of two reverse primers, DZ89 or DZ81 (5′ CGGTCGTACGCCCGCAACC 3′ or 5′ AGGATGGCCCCCTTCTGGCGTA 3′, respectively). Amplified PCR-products were analysed by agarose gel electrophoresis. Also, amplified sequences were cloned using the Perfectly Blunt Cloning Kit (Novagen). Cloned DNA was then sequenced to determine whether infections were indeed caused by mutant RNA or by RNA that had reverted to WT.

Quantitative (kinetic), real-time RT–PCR. Real-time PCR is a fluorescence-based method of quantifying PCR products during the exponential phase of amplification rather than after a fixed number of cycles. As target DNA is amplified, fluorescence increases as a result of either the binding of dsDNA to DNA stains (SYBR Green chemistry) or from the degradation of fluorogenic probes by the DNA polymerase used in the reaction [TaqMan chemistry, Perkin-Elmer (1999) and references therein]. Fluorogenic probes are oligonucleotides complementary to the region amplified by the flanking primers and are labelled with a fluorescent reporter dye (R) at the 5′ terminus and a quencher reporter (Q) at the 3′ terminus. In intact probes, the proximity of R to Q results in reduction of the fluorescence of R by Förster energy transfer through space (Forster, 1948). The generation of specific dsDNA during thermocycling leads to probe degradation via the 5′ exonuclease activity of DNA polymerase resulting in an increase in fluorescence. Therefore, the higher the initial amount of nucleic acid target in the sample, the earlier a significant increase in fluorescence is observed. Threshold cycle \( C_t \) is the point in time during cycling at which an amplification plot (representing the increase in fluorescence over time) intersects with an arbitrary threshold limit (see Figs 3 and 4). The higher the initial amount of nucleic acid in the sample the smaller the \( C_t \) value. Absolute or relative quantification is accomplished via a standard curve generated by amplifying known amounts of nucleic acids.

To quantitatively monitor the accumulation of WT- and mutant RNA in inoculated protoplasts, we used the SYBR Green RT–PCR kit (PE Applied Biosystems). The assay targeted a 114 bp region of RNA-1 (Fig. 1) amplified by primers DZ299 (5′ GAGGGTCCACCGACTAC 3′) and DZ100 (5′ TGAAATAGATAAGAACAAGAAG 3′), designed using Primer Express primer design software (PE). Typically, 1 \( \mu \)g total RNA was reverse transcribed and amplified in one tube. Reactions (30 \( \mu \)l) contained 1 \( \times \) SYBR PCR Buffer, 5.5 mM MgCl\(_2\), dNTP blend (0.5 mM dATP, 0.5 mM dCTP, 0.5 mM dGTP and 1.0 mM dUTP), 0.5 \( \mu \)M RNase inhibitor (Roche), 70 nM of each primer, Multiscribe reverse transcriptase (0.25 \( \mu \)l/\( \mu \)l) and AmpliTaq Gold DNA polymerase (0.025 \( \mu \)l/\( \mu \)l). The mixture was placed in an ABI Prism 7700 Sequence Detector and subjected to one cycle of 25 °C for 10 min and one cycle of 48 °C for 30 min for reverse transcription. DNA amplification was done using the following thermocycling programme: one cycle of polymerase activation and template denaturation at 95 °C for 10 min and 40 cycles of 95 °C for 15 s and primer annealing and extension at 60 °C for 1 min.

Also, the accumulation of WT-RNAs -1 and -2 in plants was monitored using dually labelled probes and the TaqMan Gold RT–PCR kit for RNA-1, the probe TPOL1 (6-FAM-5′ CTTCTTTATATGG-CAGGTGGTTTGCAACC 3′-TAMRA; synthesized at IDT, Corvallis, IA, USA) and primers DZ101 (5′ CAACGCCCCTCCAGGCAAT 3′) and DZ102 (5′ CGGCCAGCCAAAAA 3′) are specific to a 65 bp segment in the polymerase gene (ORF1) of RNA-1 (Fig. 1). To monitor the accumulation of RNA-2, we used probe TPOL2 (TET-5′ CCTCAAT-GGCCACCTGATCGGG 3′-TAMRA) and primers DZ109 (5′ TTT-CCTTCGGGCTTTTGGTA 3′) and DZ108 (5′ GGTGAGGACGC-TTGTCATAC 3′) to detect the amplification of a 65 bp segment of the polymerase gene (within ORF2) of RNA-2 (Fig. 1). One \( \mu \)g of total RNA extracted from newly formed leaves of plants inoculated with PEMV-1-AT + PEMV-2 at 7–10 days post-inoculation (p.i.) was amplified. Each 30 \( \mu \)l reaction contained 1 \( \times \) TaqMan buffer A, 5.5 mM MgCl\(_2\), dNTP blend (0.3 mM dATP, 0.3 mM dCTP, 0.3 mM dGTP and 0.6 mM dUTP), 0.4 \( \mu \)M dUTP, 0.8 \( \mu \)M probe, Multiscribe reverse transcriptase (0.25 \( \mu \)l/\( \mu \)l) and AmpliTaq Gold DNA polymerase (0.025 \( \mu \)l/\( \mu \)l). The thermocycling regime was as described above.
Results and Discussion

Infectivity of VPg mutants

Mutagenesis of the VPg of PEMV targeted either amino acids at the junction of the putative proteinase cleavage sites or amino acids that can potentially link the VPg to viral RNA. Based on infectivity and symptom development, mutants were categorized into three groups (Fig. 1).

Group 1 (WT-like clones): plants infected with these mutants displayed symptoms indistinguishable from WT-infections in both timing and severity.

Group 2 (attenuated clones): plants infected with these mutants displayed symptoms that were milder and appeared later than WT-infections.

Group 3 (non-infectious clones): plants inoculated with these mutants remained healthy and viral RNAs were not detected in inoculated plants by Northern blotting or by RT–PCR.

Mutants in group 1 included the mutant ET-QS in which the WT-N-terminal cleavage site (ET) was replaced by the C-terminal cleavage site (QS). This mutant was infectious in plants resulting in symptoms indistinguishable from those produced by WT-infections. Sequencing clones generated by RT–PCR of viral RNA from plants infected with ET-QS verified that the mutation was maintained in the infecting RNA. Reversion to WT-sequence was not observed in any of the clones. In contrast, mutants lacking the N- or C-terminal cleavage sites (NAET or CAQS, respectively) were not infectious in plants and their RNAs were not detected in inoculated plants by Northern blotting or by RT–PCR. These results support previous data suggesting that the functional VPg is a product of proteolytic processing by the putative 3C-like proteinase encoded upstream of the VPg region (Wobus et al., 1998). This conclusion can be further substantiated by the construction of VPg mutants in which the WT-cleavage sites (EA(T) and QS) are replaced by amino acids that maintain the T1 and the Q28 of the WT-VPg but cannot be utilized by the viral proteinase (e.g., PT or QI). Also, group 1 includes T7A, Y9S and S16A, which were infectious in plants, and no reversion to WT-sequence was observed in progeny RNA. The symptoms produced by these mutants were WT-like both in timing and in severity.

In Poliovirus 1, amino acids occupying position ‘P4’ play a critical role in regulating cleavage efficiency of the 3C-like proteinase (Wellink & van Kammen, 1988; Cordingley et al., 1990; Blair & Semler, 1991; Mirzayan & Wimmer, 1994). In PEMV, ‘P4’ is occupied by a ‘suboptimal’ threonine residue. This residue was replaced by a leucine residue in the mutant T−4L. Theoretically, this leucine residue provides optimal proteolysis. However, transcripts from T−4L were infectious resulting in mottling and chlorosis that were milder and appeared later (14 days p.i.) than those observed in WT-infections. If position P4 is shown to have a role in processing the VPg of PEMV, this reduced infectivity may indicate that suboptimal processing of the VPg provides a regulatory function. Interestingly, the phenotype of T25L, mutated in the other ‘P4’ position, was identical to that of T−4L. Similar to T−4L, this change in the context of the N-terminal cleavage site, although it has not abolished the production of a functional VPg, seems to have disrupted a balance of timing and/or rate of proteolysis resulting in an attenuated virus. In addition to NAET or CAQS discussed above, group 3 contains a set of mutations in residues that can provide the free hydroxyl side-chain required for linkage between the VPg and viral RNA. Results in Fig. 1 show that changes in the tyrosine residue (Y2) and/or the serine residue (S3) result in a nonfunctional VPg. All three mutations altering Y2 resulted in nonviable viruses. Most of the ≥ 60 plants inoculated with transcripts from each mutant (combined with WT-RNA-2 transcripts) remained symptomless and RNA-1 sequences could not be detected in those plants by RT–PCR. However, about 10% of plants inoculated with these mutants began to show very mild symptoms approximately 28 days p.i. Sequencing RT–PCR products amplified from these symptomatic plants using primers specific to the VPg region demonstrated that in these infections the mutant RNAs had reverted to WT. Clones representing the mutant RNA-1 sequences were not detected in any of these plants. The time required for reversion to the WT-sequence accounts for the delayed symptom development. This reversion was not observed when protoplasts were inoculated with these mutants. However, the relatively short incubation time of protoplasts (36 h) may have prevented the generation and recovery of the revertants observed in plants. Rapid deterioration of pea protoplasts after about 36 h prevented longer incubation times for protoplasts inoculated with VPg mutants. In the case of Poliovirus 1, extended maintenance intervals (72 h) were required before HeLa cells.
Involvement of VPg in replication of PEMV-1

Fig. 3. RT–PCR amplification plots of total RNA extracted from protoplasts infected with WT- or mutated-VPg clones. Red plot, S16A; orange, WT-RNA-1; blue, ET-Q5 (similar to plots of other clones in group 1); yellow: T—4L (similar to that of T + 4L and T25L); green, T ± 4L. Plots of clones from group 3 were identical to those of mock-inoculated or other negative controls and are represented by the black plots. Cycle number is plotted against change in normalized fluorescence ($\Delta Rn$) caused by the binding of SYBR Green to dsDNA. The SYBR Green–dsDNA complex signal is normalized by dividing the emission intensity of SYBR Green by the emission intensity of the internal, passive reference ROX. The sooner an amplification plot intersects the threshold limit (horizontal black line, usually 10$^{-\Delta}$ the standard deviation of the baseline) the higher the initial amount of RNA-1 in the sample. In this amplification, amplification of known amounts of RNA-1 transcripts indicated that a difference of four cycles reflects approximately 10-fold the amount of RNA in the initial sample.

transfected with nonviable VPg mutant RNAs produced viable revertants (Reuer et al., 1990).

Replacing S3 with an alanine also resulted in a nonviable clone (S3A). None of the 50 plants inoculated with this mutant developed any symptoms and RNA-1 sequences could not be detected in those plants by RT–PCR. Similar results were obtained when Y2 and S3 were inverted (YS2,3SY), indicating that the precise location of these two residues is important for a functional VPg. In other viruses, similar inversion of amino acids also resulted in defective viruses (Schaad et al., 1996; Reuer et al., 1990; Murphy et al., 1996). The juxtaposition of Y2 and S3 hindered the use of this mutagenesis scheme as a means of determining the linking amino acid.

**In vitro translation**

Results shown in Fig. 2 demonstrate that none of the mutations affected the in vitro translation products of RNA-1. The two major translation products (36 and 88 kDa) of WT-RNA-1 (Gabriel & de Zoeten, 1984) were also made by all the VPg mutants. This makes a qualitative role for the VPg in regulating protein expression unlikely.

**Quantitative evaluation of the replication of the VPg mutants**

To determine whether the above mentioned differences in infectivity and symptom development are attributable to reduced (or abolished) replication or to altered movement ability of the clones, we used quantitative, real-time PCR to monitor and quantify the accumulation of RNA-1 in pea protoplasts inoculated with RNA transcripts from the clones described above. Results indicate that plants infected with clones from group 1 contained RNA-1 in amounts similar to those found in plants infected with the WT-clone (Fig. 3). Also, the reduced and delayed symptoms displayed by mutants in group 2 are associated with a reduction (10- to 100-fold) in viral RNA-1 production and accumulation in infected cells. Clones of group 3 and the replicatively inactive clone pBstEII failed to replicate and accumulate to detectable levels in inoculated protoplasts. These results suggest that the inability of those clones to infect pea plants is due to their replicative, rather than movement, incompetence brought about by the mutations in the VPg.

This association between RNA level and symptom severity was also observed in pea plants displaying symptoms ranging from mild to severe despite being infected with the same isolate of PEMV (PEMV-1-AT$^+$ + PEMV-2). Results obtained by quantitative RT–PCR indicated that, invariably, higher (10- to 1000-fold) RNA-1 levels were detected in plants displaying severe symptoms compared to plants showing mild symptoms (Fig. 4a). Some severely infected plants contained up to 10000-fold the amount of RNA-1 found in plants showing mild symptoms. Even at 5 days p.i., plants showing early symptoms contained higher levels of RNA-1 compared to asymptomatic plants.

In our laboratory, we routinely use an isolate of PEMV designated PEMV-$\Delta$ for virion purification and RNA isolation. Plants infected with PEMV-$\Delta$ display earlier and more severe symptoms and yield 3- to 5-fold more virions than plants infected with other PEMV isolates (Demler et al., 1997). Pea plants infected with PEMV-$\Delta$ consistently accumulated RNA-1 to higher levels (10- to 100-fold) compared to plants infected
with PEMV-AT\(^+\) (Fig. 4\(a\)). The severity of the symptoms was dependent on the amount of RNA-1 in infected plants rather than on time p.i.

Interestingly, we could not detect a similar parallel between RNA-2 levels and symptom severity. The same plants showing great variations in symptom severity and in RNA-1 levels (Fig. 4\(a\)) had little variation in the amounts of RNA-2 (Fig. 4\(b\)). In the plants tested, the accumulation of RNA-2 was independent of RNA-1 accumulation and of symptom development. Notably, plants infected with PEMV-2 alone remain symptomless or display very mild mosaic 14–21 days p.i. Taken together, these observations suggest that symptom determinants reside in the RNA of PEMV-1.

Finally, in light of what is currently known about the symbiosis between PEMV-1 and PEMV-2, a re-examination of the findings of Reisman \\& de Zoeten (1982) leads to two conclusions. First, the VPg is covalently attached to the RNAs of both PEMV-1 and PEMV-2 and second, only RNA covalently attached to the VPg is encapsidated. These conclusions, combined with the main finding of this paper that the VPg is involved in the replication (and not in the movement) of PEMV-1, presents an interesting question: why is the VPg encoded by PEMV-1 covalently attached to the RNA of PEMV-2 in WT-infections? It is well documented that PEMV-2 can replicate in protoplasts and can infect plants systemically in the absence of PEMV-1 and the VPg encoded by PEMV-1 (Demler et al., 1996). It is intriguing to speculate that the VPg of PEMV acts as an encapsidation signal for the RNA of PEMV-2 while regulating the replication of the RNA of PEMV-1. If this dual role is proven experimentally, it will be the first instance of a VPg serving two different functions with two different viruses.
References


Received 1 October 1999; Accepted 1 December 1999