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**Detection and Diagnosis of Virus Diseases of
Pelargonium**

R.L. Jordan, A. Gera, H.T. Hsu, A. Franck, G. Loebenstein

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Investigators:
(Principal first)

Jordan, Ramon L.

Gera, Abed

Hsu, Hei-Ti

Franck, A.

Loebenstein, Gad

Affiliated Institution:

USDA-ARS, US National Arboretum, Beltsville, MD

A.R.O., Volcani Center, Bet-Dagan, Israel

USDA-ARS, US National Arboretum, Beltsville, MD

A.R.O., Volcani Center, Bet-Dagan, Israel

A.R.O., Volcani Center, Bet-Dagan, Israel

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Signature:

Principal Investigator

Ramon Jordan

Signature:

Officer of the Affiliated Institution

Thomas S. Elias, Director, US National Arboretum

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BARD Project No. US-2336-93R**FINAL SCIENTIFIC REPORT****Detection and Diagnosis of Virus Diseases of Pelargonium****Abstract**

Pelargonium (Geranium) is the number one pot plant in many areas of the United States and Europe. Israel and the U.S. send to Europe rooted cuttings, foundation stocks and finished plants to supply a certain share of the market. Geraniums are propagated mainly vegetatively from cuttings. Consequently, viral diseases have been and remain a major threat to the production and quality of the crop. Among the viruses isolated from naturally infected geraniums, 11 are not specific to Pelargonium and occur in other crops while 6 other viruses seem to be limited to geranium. However, several of these viruses are not sufficiently characterized to conclude that they are distinct agents and their nomenclature and taxonomy are confusing. The ability to separate, distinguish and detect the different viruses in geranium will overcome obstacles to developing effective detection and certification schemes.

Our focus was to further characterize some of these viruses and develop better methods for their detection and control. These viruses include: isolates of pelargonium line pattern virus (PLPV), pelargonium ringspot virus (PelRSV), pelargonium flower break virus (PFBV), pelargonium leaf curl (PLCV), and tomato ringspot virus (TomRSV). Twelve hybridoma cell lines secreting monoclonal antibodies specific to a geranium isolate of TomRSV were produced. These antibodies are currently being characterized and will be tested for their ability to detect TomRSV in infected geraniums. The biological, biochemical and serological properties of four isometric viruses - PLPV, PelRSV, and PFBV (and a PelRSV-like isolate from Italy called GR57) isolated from geraniums exhibiting line and ring pattern or flower break symptoms - and an isolate of elderberry latent virus (ELV; which the literature indicates is the same as PelRSV) have been determined. Cloned cDNA copies of the genomic RNAs of these viruses were sequenced and the sizes and locations of predicted viral proteins deduced. A portion of the putative replicase genes was also sequenced from cloned RT-PCR fragments. We have shown that, when compared to the published biochemical and serological properties, and sequences and genome organizations of other small isometric plant viruses, all of these viruses should each be considered new, distinct members of the Carmovirus group of the family Tombusviridae. Hybridization assays using recombinant DNA probes also demonstrated that PLPV, PelRSV, and ELV produce only one subgenomic RNA in infected plants. This unusual property of the gene expression of these three viruses suggests that they are unique among the Carmoviruses.

The development of new technologies for the detection of these viruses in geranium was also demonstrated. Hybridization probes developed to PFBV (radioactively-labeled cRNA riboprobes) and to PLPV (non-radioactive digoxigenin-labeled cDNAs) were generally shown to be no more sensitive for the detection of virus in infected plants than the standard ELISA serology-based assays. However, a reverse transcriptase-polymerase chain reaction assay was shown to be over 1000 times more sensitive in detecting PFBV in leaf extracts of infected geranium than was ELISA.

This research has lead to a better understanding of the identity of the viruses infecting pelargonium and to the development of new tools that can be used in an improved scheme of providing virus-indexed pelargonium plants. The sequence information, and the serological and cloned DNA probes generated from this work, will allow the application of these new tools for virus detection, which will be useful in domestic and international indexing programs which are essential for the production of virus-free germplasm both for domestic markets and the international exchange of plant material.

Objectives and Approaches of the Original Proposal

Objectives: Develop improved methods for the detection and diagnosis of viruses of pelargonium based on improved serological methods and for the detection of viral genomes in pelargonium utilizing recombinant DNA technologies. Characterize domestic and foreign pelargonium virus isolates and determine existence of virus strains. Apply and evaluate the use of improved detection methods for use in existing nursery certification schemes for providing virus-indexed pelargonium plants.

Specifics: (1) The first objective of this research was to develop improved methods for the detection and diagnosis of previously characterized viruses including pelargonium flower break carmovirus (PFBV), pelargonium line pattern virus (PLPV), and tomato ringspot nepovirus (TomRSV). Antisera to these viruses were to be acquired from other researchers where available or will be produced as required. Emphasis will be placed on increasing the sensitivity and reliability of the assays from pelargonium. Improved methods of detection of virus-specific nucleic acids utilizing cDNA or cRNA nucleic acid probes and/or the polymerase chain reaction (PCR) would also be investigated for several of these viruses. (2) The second objective of this proposal was to obtain and/or isolate and characterize pelargonium ringspot virus (PelRSV) and pelargonium leaf curl tombusvirus (PLCV) isolates from pelargonium from the U.S., the U.K., Europe, and Israel. Antisera to these isolates would be acquired from other researchers where available or will be produced as required. (3) The third objective deals with applying and evaluating the developed reagents and procedures in industry/nursery certification indexing schemes for providing virus-indexed pelargonium plants.

Approaches: Isolate and characterize domestic and foreign pelargonium virus isolates, characterize virions, viral coat protein, and viral nucleic acids (ss and dsRNAs), and determine existence of virus strains. Develop improved detection methods for viruses in pelargonium nuclear stock plants based on improved immunological methods using polyclonal and monoclonal antibodies, and on methods for the detection of viral genomes in pelargonium utilizing cDNA probes, cRNA probes (riboprobes) and the polymerase chain reaction (PCR and immunocapture-PCR). Apply and evaluate the use of these improved detection methods in existing nursery certification schemes for providing virus-indexed pelargonium plants. The focus of this work will initially be concerned with pelargonium line pattern virus and tomato ringspot virus, but the pelargonium viruses pelargonium flower break, pelargonium ringspot, and pelargonium leaf curl viruses will be targeted as well.

FINAL SCIENTIFIC REPORT

**Detailed description of the research activities and results,
evaluation of the research accomplishments, description of the cooperation, and conclusions**

Relevant Data, Methodology, Results and Discussion

I. Introduction

Pelargonium (Geranium) is the number one pot plant in many areas of the United States and Europe. Israel and the U.S. send to Europe rooted cuttings, foundation stocks and finished plants to supply a certain share of the market. Geraniums are propagated mainly vegetatively from cuttings. Consequently viral diseases have been and remain a major threat to the production and quality of the crop. With the foreseen change in the EC regulations a new scheme for the production of certified pathogen-tested material of pelargonium has been proposed. Included in the list are 8 viruses that require certification. These viruses can cause severe economic losses by reducing vegetative growth and plant quality.

Separating and distinguishing the different viruses in pelargonium has presented a major problem in developing an effective certification program. Furthermore, there is confusion about the exact identity of several of the different viruses described, especially with regard to pelargonium ringspot virus.

Our research intentions were to develop better detection methods for viruses in pelargonium, based on improved immunological methods using polyclonal and monoclonal antibodies, with emphasis on tomato ringspot virus, pelargonium ringspot, pelargonium flower break, pelargonium line pattern and pelargonium leaf curl viruses and to determine existence of virus strains. We also were determined to characterize the viral nucleic acids and develop methods for detection of the viral genome in pelargonium utilizing cDNA probes, riboprobes and the polymerase chain reaction.

The results of this research should lead, therefore, to a better understanding of the identity of the viruses infecting pelargonium and to an improved scheme of providing virus-indexed pelargonium plants.

II. Summary of Results from First and Second Annual Reports

A. Tomato ringspot virus (TomRSV) {US National Arboretum, Beltsville}

1. Domestic isolates (California, Ohio and Pennsylvania) were obtained, either as TomRSV-infected geranium or as TomRSV-infected *Nicotiana benthamiana*. Isolates were transferred to other suitable host range indicator plants and tested by serology to confirm virus identity and status. Polyclonal antisera from American Type Culture Collection (Rockville, MD) and Agdia, Inc (Elkhart, IN) made against woody host isolates of TomRSV have been tested against the domestic pelargonium isolates. In ELISA tests these

antibodies do react with the geranium isolates. A monoclonal antibody (as ascities fluids) reactive against a geranium isolate of TomRSV has been obtained from C. Powell (Univ Florida, Ft. Pierce). Evaluation of the reactivity of this monoclonal antibody against the above isolates were determined.

2. The titers of TomRSV in inoculated cucumbers were monitored DAS-ELISA daily for two weeks. Concentration of TmRSV in inoculated cucumber cotyledons reached the peak on the 8th day post inoculation and declined sharply on the 9th day and thereafter. The titers of the virus in roots and true leaves were insignificant comparing the peak titer in cotyledons 8 days after inoculations. Therefore, inoculated cotyledons were used as sources of virus purification. A method for purification of TomRSV infecting geranium was developed. Virus infected cucumber cotyledons were homogenized in K-citrate buffer (pH 6.5) containing thioglycolic acid and clarified by low speed centrifugation. Supernatants were subjected to high speed centrifugation to pellet the virus. Pellets were resuspended in phosphate buffered saline solution (PBS, pH 7.2), and were given a cycle of low speed, and high speed differentiation centrifugation. Following resuspension of the pellets in PBS containing a non-ionic detergent, the virus suspension was clarified at low speed and pelleted through a sucrose cushion by high speed centrifugation before loaded on a sucrose density gradient. Virus bands were collected after centrifugation. Sucrose was removed by dialysis or high speed centrifugation. Yields of TomRSV ranged from 0.232 to 0.488 mg with an average of 0.353 mg per 100 g tissue. These preparations were used to produce polyclonal and monoclonal antibodies.

3. Inoculation of geranium was made with purified preparations of TomRSV. Symptoms included chlorotic rings about 2 mm and concentric chlorotic rings of 4 to 5 mm in diameters. Chlorotic irregular line patterns were also observed.

B. Pelargonium line pattern virus (PLPV) {US National Arboretum, Beltsville}

1. The biological, biochemical and serological properties of a virus isolated from *Pelargonium x hortorum* exhibiting line and ring pattern symptoms have been determined and compared with pelargonium ringspot virus (see C below).

2. PLPV RNA was isolated from purified virus preparations and used to produce a cDNA library using reverse transcriptase and random primers. *EcoRI* adapters were ligated to the cDNAs. The fragments were then purified and ligated to *EcoRI*-digested pSK plasmid vector. XL-1 Blue bacteria were transformed with the plasmid and putative transformants were screened with polyclonal anti-PLPV antisera. Coat protein-expressing clones were prepared for miniprep analysis and screened by gel electrophoresis. Two clones with about 400 bp inserts was selected for sequencing. The sequence of these two coat protein clones will be described with the rest of the viral sequences below in section III.A.

3. A reverse transcriptase-polymerase chain reaction (RT-PCR) fragment of approximately 418nt was amplified from both virus-specific dsRNA and from total nucleic acids preparations extracted from PLPV-infected *C. quinoa*. The primer pairs used for RT-PCR amplify a highly conserved region within viral RNA-dependent RNA-polymerase (RdRp) genes from carmo-like viruses (includes carmoviruses and dianthoviruses). This fragment has been cloned (as above) and sequenced. Comparisons to sequences of other carmo-like viruses, including PelRSV, will also be described below in section III.A.

C. *Pelargonium* line pattern (PLPV) and ringspot virus (PelRSV) comparisons {US National Arboretum, Beltsville}

1. Virus isolates: PLPV was isolated from a naturally-infected ornamental geranium (*Pelargonium x hortorum*) variety 'Crimson Fire' exhibiting line and ring pattern symptoms (see Kinard *et al*, 1996). The isolate (PLPV-CF) was originally transferred to *Chenopodium quinoa* Willd. from geranium leaves using 0.01 M Tris-Cl, pH 7.3 containing 2% PEG 6000. Also used in this study was an isolate from Denmark (PLPV-D) provided by N. Paludan (Langby, Denmark) and maintained in *C. quinoa*. The PelRSV isolate was generously provided by M. Schönfelder (Braunschweig, Germany) and maintained in *C. quinoa* using 0.05 M Tris-Cl buffer, pH 7.6 for transfers.

Purified PLPV-D was inoculated to seven different PLPV-free (as determined by ACP-ELISA) *Pelargonium x hortorum* cultivars. PLPV could be detected by ELISA in the cultivars 'Geronimo', 'Snowden Improved', and 'Sprinter Scarlet' within 30 days after inoculation. PLPV could also be recovered from geranium leaves by inoculations to *C. quinoa* made at about 55 days post-inoculation. Within 50-80 days after inoculation, symptoms consistent with those observed on the original 'Crimson Fire' geranium were visible on the three cultivars. Inoculation of geraniums with PelRSV is in progress. On *C. quinoa* both PLPV and PelRSV caused diffuse chlorotic local lesions, visible 5-8 days post-inoculation, that progressed to necrosis. No systemic symptoms were observed on *C. quinoa* with either virus. Geranium cultivars inoculated with purified PelRSV preparations have yet to show symptoms, but were shown to be infected using hybridization assays (see section III.B.).

2. Purification: Virions were purified from inoculated *C. quinoa* leaves harvested 5-8 (PLPV-CF, PLPV-D) or 10-14 days (PelRSV) post-inoculation. Leaf tissue was homogenized in 4 vol. (v:w) 0.05 M KPO₄, pH 8.0, containing 0.01 M DIECA and 0.02% 2-mercaptoethanol. For PLPV the homogenate was clarified by low-speed centrifugation (3,300 x g). A suspension of bentonite (40 mg ml⁻¹) was included in clarification of the PelRSV homogenate; small aliquots of bentonite were added, stirred, and removed by centrifugation (7,800 x g) until the supernatant was straw-colored. For both viruses particles were then precipitated by the addition of NaCl to 0.16 M and PEG 6000 to 9.2% with stirring at 4°C (2 h) followed by centrifugation at 24,000 x g (20 min). Pellets were resuspended in 0.01 M Tris-Cl, pH 7.3 (Tris buffer), clarified by low-speed centrifugation, and for PLPV only, pelleted by high-speed centrifugation at 109,000

x g for 1.5 h. Virions suspended in Tris buffer was sedimented through 10-30% linear-log sucrose gradients for 2 h at 40,000 rpm in an SW 41 rotor. Virus-containing fractions were removed and concentrated using Amicon-30 Centricon devices with Tris buffer. An extinction coefficient at 260 nm of $6.5 \text{ cm}^2 \text{ mg}^{-1}$ was used to estimate concentrations. Purified preparations were stored at -135°C .

The procedures used for purification were developed for PLPV and applied, with minor modifications, to PelRSV. Yields of PLPV were greater when purifying from freshly harvested *C. quinoa* than from tissue that had been frozen. Recovery of infectious particles was significantly reduced when procedures included extraction above 4°C , detergents (2% NP-40, 2% Triton X-100, 1% SDS), buffers of $\text{pH} < 7.0$, EDTA-containing buffers, or high molarity (0.2 M) phosphate buffer. The absorbance ratio at 260/280 nm for purified preparations of both PLPV and PelRSV was 1.60-1.90. Intact spherical particles were observed for both viruses in transmission electron microscopy using 0.25% uranyl acetate as a negative stain (see Kinard *et al*, 1996). Particle dimensions for both PLPV and PelRSV were about 30-32 nm.

3. RNA analyses: Encapsidated RNA was released by heating purified virions in 40 mM Tris-Cl, pH 9.0, containing 2 mM EDTA, 1 mg ml^{-1} fractionated bentonite, and 2 % SDS to 56°C for 15 min. RNA was analyzed by electrophoresis through 1.0% agarose gels, submerged in 1 X TAE, and subsequently stained in solution of $0.5 \mu\text{g } \mu\text{l}^{-1}$ ethidium bromide. RNA standards (BRL) were included to estimate the molecular weights of the viral RNAs. Double-stranded RNA (dsRNA) was extracted from inoculated and uninoculated plants and analyzed essentially as described by Jordan *et al*. (1983).

Purified PLPV preparations contain two species of RNA migrating at 1.5 and $0.5 \times 10^6 M_r$ and PelRSV contains a single species of RNA migrating at $1.6 \times 10^6 M_r$ (not shown). Two dsRNA species of about 3.1 and 1.1, and 3.3 and $1.2 \times 10^6 M_r$ were detected in *C. quinoa* infected with PLPV and PelRSV, respectively (see Kinard *et al*, 1996). An illustrated comparison of the PLPV induced dsRNA pattern with those of several other small, isometric RNA-containing viruses, including PFBV, is also shown in Kinard *et al*, 1996. The distinct dsRNA patterns exhibited in *C. quinoa* infected with CarMoV, CRSV, PLPV, and PelRSV are also illustrated.

4. Coat protein analysis: Viral coat proteins were analyzed with discontinuous SDS-PAGE using 5% stacking and 12.5% separating gels. Virions were dissociated in 0.06 M Tris-Cl, pH 6.8, containing 2% SDS, 5% 2-mercaptoethanol, and 15% glycerol by heating to 95°C for 5 min. Electrophoresis was at 80 V for 30 min, followed by 150 V for 1.5 h and protein bands were stained with 0.1% Coomassie brilliant blue R-250. Standards (Sigma) were included and used to estimate the molecular weight of the proteins.

SDS-PAGE revealed a single protein of 36-37 kDa for both PLPV and PelRSV (see Kinard *et al*, 1996). Protein samples of the two viruses essentially co-migrated, although in some experiments PLPV protein was observed to have a slightly reduced M_r ($< 500 \text{ Da}$ difference) compared to PelRSV.

5. Serology: Antisera to PLPV-D were produced in New Zealand white rabbits that were immunized with a total of 0.5 mg purified (equal mixture of native and denatured) virus injected intramuscularly in aliquots over 35 days. Sera were collected at 45 and 60 days after the first inoculation. Antigen-coated plate (ACP-) and double-antibody sandwich (DAS-) ELISAs were performed as described (Jordan and Hammond, 1991). Antisera to PFBV was obtained from Agdia (Elkhart, IN). Antisera to PLPV-N (Bouwen and Maat, 1992) was also obtained from Agdia as a non-commercial sera test sample. Antisera to PelRSV (Bouwen and Maat, 1992) was obtained from A. Brunt (Horticulture Research International, Littlehampton, UK). Other viral antigens and antisera were obtained from ATCC (Rockville, MD): carnation mottle virus (CarMoV; PV-108, PVAS-108); CRSV (PV-21, PVAS-21); cucumber mosaic virus (CMV; PV-242, PVAS-242a); and PFBV (PV519).

Table 1 in Kinard *et al*, 1996 (not repeated here) presents the results of ACP- and DAS-ELISA tests of PLPV-CF, -D, -N, and PelRSV against antisera to the PLPV-D, and -N isolates and PelRSV. PLPV and PelRSV are serologically distinct in these tests. No serological relationships were revealed among any PLPV isolate or PelRSV and the following viruses using ACP-ELISA with polyclonal sera: Broad bean wilt virus, CarMoV, CMV, CRSV, PFBV, southern bean mosaic virus, four tomosviruses, or seven comoviruses (data not shown).

6. A reverse transcriptase-polymerase chain reaction (RT-PCR) fragment of approximately 418nt has been amplified from total nucleic acids preparations extracted from PelRSV-infected *C. quinoa*. The primer pairs used for RT-PCR amplify a highly conserved region within viral RNA-dependent RNA-polymerase (RdRp) genes from carmo-like viruses (includes carmoviruses and dianthoviruses). The template for reverse transcriptase polymerase chain reaction (RT-PCR) was dsRNA extracted from plants inoculated with PelRSV and PLPV. A mixture of dsRNA (1-2 µl) and random hexamers (2.5 µM) was boiled for 5 min. immediately after which a reverse transcription cocktail (10 ul) of 50 mM KCl, 10 mM Tris-Cl, pH 8.3, 5 mM MgCl₂, 1 mM each deoxynucleoside triphosphate, 1 U RNAase inhibitor, and 2.5 U M-MLV reverse transcriptase was added. The mixture was incubated through a single cycle of 42°C for 30 min, 99°C for 5 min, and 4°C for 5 min. For PCR, an additional cocktail (40 ul) containing 50 mM KCL, 10 mM Tris-Cl, pH 8.3, 2 mM MgCl₂, 2.5 U Taq DNA polymerase, and 0.2 µM each primer was added. The primers used in the PCR reaction (Morozov *et al*, 1995): AARGTCGACCCGWNCCNMGNGTNATHCAAACC (upstream), GNMCTGCAGNACRCARTCRTCNCCRTTRTT (downstream) target conserved sequences within the replicase-encoding genes of carmo-like viruses. The PCR was performed with a regime of an initial cycle of 94°C for 1 min; 35 cycles of 1 min at 94°C, 3 min at 48°C, and 3 min at 72°C; and a final cycle of 10 min at 72°C. The amplification products were analyzed by electrophoresis through 1.2% agarose gels, submerged in 1X TAE, that were subsequently stained in a solution of 0.5 µg ml⁻¹ ethidium bromide.

This fragment has been cloned (as above) and sequenced. Comparisons to sequences of other carmo-like viruses, including PLPV, show similarities of only 28-40% with the published sequences of carmoviruses, tomosviruses and dianthoviruses (see section III.A. below).

D. Preparation and utilization of a riboprobe to pelargonium flower break virus (PFBV) {Volcani Center, Bet-Dagan}

1. The preparation of the riboprobe was based on the following steps: Viral RNA was obtained from highly purified virus, using phenol-SDS extraction and removal of DNA traces by DNAase treatment. cDNA was prepared to viral RNA using reverse transcriptase and random primers. The cDNA was blunt end-ligated into pUC19 vector after cutting the pUC19 with Sma I restriction enzyme. JM109 bacteria were transformed with the plasmid and after preparation of minipreps the clones were screened by gel electrophoresis. A clone with about a 3500 bp insert was selected. The insert was radiolabelled and tested by hybridization analysis with PFBV-RNA and control RNA obtained from other viruses. A specific 1600 bp insert cut out from the 3500 bp fragment was then subcloned in pGEM-7Z(+) (Promega), containing SP6 and T₇ promoters. A transcript was obtained using T₇ RNA polymerase which hybridized in dot spot and Northern blots with PFBV RNA, but not with RNA from pelargonium leaf curl virus (PLCV).

2. This probe was able to detect PFBV in extracts of infected pelargonium plants. A positive reaction was obtained (40 h exposure) with a plant extract dilution of 1:6400, while in comparable ELISA tests virus was detected at a plant extract dilution of 1:1600. Infected pelargonium plants were obtained by grafting 4 wk old plants with scions from PFBV infected pelargonium shoots. One batch of infected plants was transferred to a controlled-temperature chamber maintained at 29°C, continuously. A second batch of plants was placed in a chamber at 20°C. Tissue disks 10 mm in diameter were taken from the upper and lower leaves several times after graft-inoculation. Virus titer were compared by ELISA and dot blot hybridization. For dot-blot, disks were triturated in 1 ml of 200 mM di-potassium phosphate containing 0.1% Triton X-100, 5 mM dithiothreitol (DTT) and 10 mM 2-mercaptoethanol (ME). Extracts were centrifuged and supernatant was added to an equal volume of denaturation solution (8X SSC + 10% formaldehyde) and incubated for 15 min at 60°C. Crude extracts were diluted in 4X SSC + 5% formaldehyde. 2 µl of the diluted extracts was spotted onto Nylon membrane and baked at 80°C under vacuum for 30 min. Membranes were prehybridized for 3 h at 42°C in the presence of 50% formaldehyde according to the membrane manufacturer's instructions (Gelman); subsequently, 105 cpm/ml ³²P-uridine-labeled cRNA transcript was added and membranes were hybridized overnight at 42°C. Membranes were washed with 1X SSC containing 0.1% SDS for 15 min at 42°C and twice with 0.1X SSC containing 0.1% SDS for 15 min at 65°C. Membranes were rinsed twice in 2X SSC, after which 0.2 µg/ml RNase A was added and incubated for 15 min at room temperature. The final wash was in 0.1X SSC containing 0.1% SDS for 30 min at 50°C. Membranes were exposed to X-ray film for 24-72 h. For ELISA, plant extracts were prepared by grinding leaf samples (1g/10 ml buffer) in 0.1 M Tris (hydroxymethyl) aminomethane-HCL buffer, pH 8.6, containing 0.2% Tween-20, 0.2% polyvinylpyrrolidone and 0.4% bovine serum albumin. Plant sap was diluted as for the dot blot hybridization. The plates were coated with IgG at a concentration of 1 µg/ml. The PFBV alkaline phosphatase conjugate was used at a dilution of 1:1,000. A negative control consisting

of crude sap from healthy plants was included in all assays.

Approximately 10 pg/ml of viral RNA could be readily detected in dot-blot hybridization, when purified viral RNA was tested. PFBV could still be detected in infected pelargonium tissue after diluting the samples 1:3200. No detectable signals were observed with sap from healthy pelargonium plants. Similar results were obtained by ELISA. The riboprobe and ELISA were powerful means for the detecting PFBV in pelargonium plants kept at the various temperature. When two batches of graft-inoculated pelargonium plants were transferred to and maintained at 20° and 29°C, continuously and plants were sampled 2, 4 and 8 weeks after inoculation, PFBV could still be detected in infected plants after diluting the samples 1:3200 when tested by ELISA and/or dot hybridization. Virus concentration was low in Pelargonium plants kept at 29°C in comparison to 20°C. However, the limits of detection of the riboprobe and ELISA were similar. The cRNA probe was further used for studying the distribution of PFBV in graft-inoculated Pelargonium plants. The riboprobe consistently detected PFBV in nearly all leaves throughout the grafted plants, three weeks after graft-inoculation. The virus was first detected in the upper leaves 17 days post graft-inoculation (DPGI) and later, on the lower leaves, 21 DPGI.

E. Development of RT-PCR for PFBV detection {Volcani Center, Bet-Dagan}

1. RNA extraction: An efficient RNA extraction procedure is crucial for successful nucleic acid amplification. Four extraction methods were initially evaluated with Pelargonium tissue: (i) phenol extraction at room temperature and not at 80°C; (ii) lithium chloride, NP-40 and sodium deoxycholate extraction (lithium method), (iii) GeneReleaser (Bio Ventures, Inc., Murfreesboro, TN, USA) and (iv) TRI reagent (Cincinnati, OH, USA). Intact ribosomal RNA was extracted, with various degrees of success, from leaf pelargonium tissue by all methods. Of the four methods, the lithium based method (which avoids phenol) was selected. This decision was based on comparing the PCR products obtained with nucleic acids extracted with these methods, cost of reagents and safety to user and environment. Tissue (100-200 mg) was homogenized in a roller press (Elektrowerk, Bhncke & Co., Hannover, Germany) with 2.5 volumes of buffer (200 mM Tris-Cl, pH 8.5, containing 1.5% lithium dodecylsulphate, 300 mM lithium chloride, 10 mM EDTA, 1% sodium deoxycholate, 1% NP-40) and 0.5% 2-mercaptoethanol. The extract was collected in a 1.5 ml microfuge tube, an equal volume of 6M potassium acetate, pH 6.5 was added and the mixture centrifuged at 14,000xg for 10 min. Nucleic acids were precipitated from the supernatant with isopropanol and centrifuged as above. The pellet was washed with 70% ethanol, dried vacuum and resuspended in 25 µl of sterile water. The lithium method was found suitable for rosaceous plant materials and has since been used in this study.

2. Oligonucleotide primers: The primers were selected from a central region of the RNA, within the open reading frame of the putative RNA polymerase. The sequences used lie over regions that are conserved within the carmoviruses. The following primers were used: U1, 5'-CGTATACTGGCTCCTCTGC-3'; L1,

5'- CACAGTTGTTACCTTGCC-3'; L2, 5'-CGCTGGACAAGATCTGGG-3'; L3, 5'-CCCTGTCCGGAAGGGG-3'. The primers were designed to amplify 343, 510 and 832 bp fragments from PFBV-RNA, respectively. The primer pairs U2 and L4 with the sequences 5'-ACCCAGTACCCGCGGGCC-3' and 5'-ACCTGCATCCCGTACAGC-3', flanking a 328 base portion of the replicase gene were also used.

3. Reverse transcription and PCR amplification: Early RT-PCR experiments were done in a two step reaction - (1) cDNA synthesis, (2) amplification. For cDNA synthesis, viral- or total plant-RNA (2 µl) was added to 18 µl of reverse transcription (RT) reaction mix: primers (0.4 M), 2 µl of first strand synthesis buffer (supplied with the RT enzyme), 1 µl of all four deoxynucleotides mixture (2.5 mM each), 1 µl DTT (100 mM) and 1 µl MMLV reverse transcriptase (Biolabs 25 u/µl) and water to a final volume of 20 µl. The reaction mixture was incubated at 37°C for 60 min. The PCR reaction mixture was 1 mM with respect of MgCl₂. Taq polymerase was used according to the manufacturer's recommendations (Stratagene, La Jolla, CA, USA). The reaction mixture was layered with 30 µl of mineral oil and then subjected to 35 cycles of amplification in a thermocycler (Hybaid, UK). Each cycle consisted of 1 min at 96°C, 2 min at 54°C and 3 min at 72°C. Samples (5 µl) of PCR products were analyzed by electrophoresis in a 1.5% agarose gel which was subsequently stained with ethidium bromide (EtBr). In later experiments, the RT and PCR reactions were done in a single non-interrupted thermal cycling program. Each reaction contained the RNA template (about 1 µg), virus specific primers (0.4 µM), 250 µM dNTPs, 25 mM MgCl, 2.5 µl of 10X reaction buffer giving a final concentration of 67 mM Tris-HCl, pH 8.8, 17 mM (NH₄)₂SO₄, 1 mM ME, 6 µM EDTA and 0.02% Gelatin. Two units of Taq DNA polymerase (Advanced Biotechnologies, UK) and 5 unit MMLV RT (Promega) were added to each reaction. The total volume was 50 µl which was overlaid with 50 µl of mineral oil and incubated in a thermocycler (Hybaid, UK) according to the following protocol: 30 min at 42°C, 3 min at 95°C, then 40 cycles of 92°C for 30 sec, 60°C for 30 sec and 72°C for 1 min, then finally 5 min at 72°C. PCR products were electrophoresed in a 1.2% agarose gel and stained with EtBr. All selected primers worked well in the preliminary test, giving the predicted size of the amplified fragments (Fig. 2). The products of the RT-PCR, using the primer pairs U1-L1, U1-L2 and U1-L3 gave distinctive bands of about 350, 500 and 850 bp fragments when PFBV-RNA was used as a template, respectively. The product of the RT-PCR, using the primer pairs U2 and L4, gave a band of about 330 bp when total RNA extracts made from pelargonium plants, infected with PFBV (data not shown). No bands were detected when healthy plant RNA extracts or in negative controls in which either primers or RNA were omitted. The size of band observed in all cases was the same, when compared with molecular weight markers.

F. Pelargonium leaf curl virus (PLCV) {Volcani Center, Bet-Dagan}

1. PLCV was purified and an antiserum was prepared. The antiserum had a titer of 1:10,000 in ELISA against PLCV-infected *Nicotiana clevelandii*. This antiserum gave good results with a PLCV isolate from

East Germany (tested there) and medium results with PLCV isolates from Holland (tested there). Our virus gave good results with an antiserum obtained from Denmark and poor results with an antiserum obtained from Italy. This data suggests a serological difference between geographically different PLCV isolates.

2. The molecular weight of the coat protein of our PLCV isolate was determined to be ~37.5 KDa.

3. The incidence of PLCV in Israel seems to be very limited and only a very few PLCV-infected plants were found.

G. Other Disorders {Volcani Center, Bet-Dagan}

1. Vein netting: Attempts to obtain ds-RNA from vein netting symptomatic plants were unsuccessful (i.e., no dsRNAs were detected).

2. Pelargonium plants with ringspot: Plants exhibiting ringspot-like symptoms were located in a nursery in December 1994. The plants were serologically tested and did not contain PFBV. Tests with the following antiserum were also negative: pelargonium ringspot virus, tomato ringspot virus and tobacco ringspot virus.

3. During the 1996 Winter season the following disorders were noted: (A) Mosaic on leaves of *P. peltatum* var. Minicascade. (B) Deformation and color breaking of petals in *P. zonale*. These symptoms were not transmissible by grafting to healthy pelargonium plants. No viral particles were detected by electron microscopy.

III. Methodology, Results and Discussion from Research During Year Three of the Project

A. Cloning, sequencing and genome organization of PLPV, PelRSV and ELV {US National Arboretum, Beltsville}

1. Methods

Virus isolates. PLPV was as described above. PelRSV was kindly provided by M. Schonfelder (DSM, Germany). ELV was obtained from ATCC (PV 522, Rockville, MD) as contributed by A.T. Jones (SCRI, Scotland). All were maintained in and purified from *C. quinoa* using 0.05 M Tris-Cl, pH 7.6, as the inoculation buffer.

Virus purification and RNA extraction. Virions were purified as previously described (Kinard et al. 1996). Briefly, leaf tissue was homogenized on 0.05 M KPO₄, pH 8.0 containing 0.01 M DIECA and 0.02% mercaptoethanol. Clarification was achieved by stirring small aliquots of 40 mg/ ml bentonite, followed by 3,300 x g centrifugation. Virions were precipitated by the addition of PEG 6000 to 9.2%, resuspended, and

sedimented through 10-30% sucrose gradients at 101,925 x g for 2 h. Viral RNA was extracted from purified virus using TriZol total RNA extraction reagent (Life Technologies). Double stranded RNA (dsRNA) from infected *C. quinoa* was extracted according to the methods of Jordan et al. (1983).

cDNA synthesis and cloning. First strand cDNA synthesis was initiated using random primers and second strand synthesis was performed using DNA polymerase I from RNAase H nicked hybrid. EcoRI/NotI adaptors were added to the cDNA, which was then ligated into EcoRI digested pUC18 and used to transform INV Φ F' competent *E. coli* cells. Cells were induced for fusion protein expression by the addition of 20 mM IPTG and lysed in ELISA plates by the addition of 5X carbonate buffer, pH 9.0, and one cycle of freezing/thawing. The PLPV and PeIRSV cDNA libraries were screened for epitopes recognized by PLPV and PeIRSV polyclonal antisera, respectively, using indirect antigen-coated ELISA. Several PLPV and one PeIRSV specific clone were identified by this method. No ELV clones were identified using PeIRSV antisera (PeIRSV antisera does react with ELV).

Nucleotide sequencing. Clones selected for sequencing by size and/or restriction digestion profiles were grown overnight in 2X YT media and plasmid was extracted using RPM preps (Bio 101). Automated sequencing was performed in both directions using M13F, M13R, or virus sequence-specific primers with dye terminators and an ABI 310 Genetic Analyzer.

Polymerase chain reaction. With PeIRSV only, two gaps in continuity, ultimately determined to be 26 and 84 nucleotides, remained after the overlapping clones were sequenced. These gaps were filled in with clones generated by RT-PCR using 23mer sequence specific primers. Reverse transcription of viral RNA was with the downstream specific primer, followed by 35 cycles of PCR (94 °C-1 min, 62 °C - 2 min, 72 °C-2 min) with both primers. The PCR products were cloned into TA vector (Invitrogen) and sequenced as previously described.

Sequence determination of termini. To confirm the 3' terminal nucleotides, viral RNA was poly A tailed using Poly A polymerase (Promega). cDNA, the synthesis of which was initiated using oligo-dT-dT primer, was cloned and sequenced as described above. To determine the 5' termini, cDNA synthesis was initiated by virus-specific primers; the cDNAs were cloned and sequenced as described above.

Analyses of sequence data. Nucleotide and amino acid sequences were assembled using Autoassembler (ABI) and analyzed using GeneJockey II (Biosoft) or University of Wisconsin Genetics Computer Group (GCG 9.7) software. Pair-wise comparisons were performed using GAP with adjusted alignment scores determined from 25 randomizations. Determination of virus-related sequences were initially identified using the BLAST program of GenBank.

Northern hybridizations. Northern blots of viral genomic and dsRNA, and total nucleic acid from infected *C. quinoa* were probed with digoxigenin-labeled 5' and 3' PLPV-, PeIRSV- and ELV-specific probes. The probes were generated by labeling, with digoxigenin (Boehringer Mannheim), the appropriate 5'- and 3'-specific clones using PCR with M13F and M13R primers. Samples were separated on 1% agarose gels under denaturing conditions and transferred to Nytran membranes (S&S), all using standard procedures (Sambrook et. al, 1989). After overnight hybridization at 60°C, membranes were rinsed in water and then

washed twice for 15 min at room temperature in 2 X SSC and 0.1% SDS, followed by two 15 min rinses at 65°C in 0.1 X SSC and 0.1% SDS. Anti-digoxigenin alkaline phosphatase-labeled antisera bound to target was detected colorimetrically using NBT and BCIP.

2. Results

Analysis of viral RNA. In agarose gels, purified viral RNA from PLPV, PelRSV and ELV resolved into two species, one prominently displayed at about 4 kb and a less prominent species at about 1.5 kb. The RNAs of these viruses were infectious when inoculated to *C. quinoa* (data not shown). Clones representing the 5' and 3' regions of these viruses were used to generate, with PCR, dig-labeled probes. All three sets of probes were observed to be virus specific. That is, the probes did not hybridize to viral RNAs nor the dsRNAs from the other viruses (not shown). Also, none of the probes hybridized to the purified dsRNAs of other carmo-like viruses including pelargonium flower break virus (PFBV), carnation mottle virus (CarMV), and a potentially new geranium virus described by Lisa et al. (1996) currently called GR57 (not shown).

To determine whether the RNAs were present in infected tissue, dsRNA and total nucleic acid extracted from *C. quinoa* were probed. The 5' specific probes hybridized only to the upper bands of the homologous virus-specific RNA, dsRNA, and *C. quinoa* extracts. The probe did not hybridize to extracts from mock inoculated *C. quinoa* or to the other viral-specific RNAs. The 3' probes, however, hybridized to both upper and lower bands of homologous virus-specific RNAs, but again not to mock inoculated *C. quinoa*, nor to the other viral RNAs (Fig III.A.1. below). These data indicate that the smaller RNAs of PLPV, PelRSV and ELV are viral and originate from the 3' terminus of the genomes. Thus, they are likely subgenomic (sg) RNA species.

cDNA synthesis and cloning. The PLPV-, PelRSV- and ELV-specific clones contained inserts ranging from 0.2-1.7 kb. These included ~419 nt clones that were amplified from PLPV and PelRSV viral RNAs using a carmovirus RdRp specific primer pair (Morozov et al., 1995). In addition, in the case for PLPV and PelRSV, several clones were selected that expressed epitope(s) recognized by PLPV or PelRSV polyclonal antisera. No ELV-specific clones were obtained that expressed epitopes recognized by PelRSV polyclonal antisera, nor were amplification products obtained from ELV viral RNA or dsRNA when using the RdRp primer pair. Once the overlapping clones were sequenced, one to two gaps interrupted the continuity. These were filled using sequence specific primers with RT-PCR. In all cases, the non-gap sequence was identical to that already obtained from cDNA clones. Including those used to sequence the termini, all nucleotides comprising the PLPV, PelRSV and ELV genomes have been determined from at least two clones.

Determination of terminal sequences. The sequence of the 5' terminus of PelRSV was determined by initiating cDNA synthesis with a primer complementary to nt 356-378. All the clones (3) that were sequenced began with the same nucleotides (TCTCA...). For ELV, 3 clones that began with the same nucleotides (CGCTT...) and four other clones that began within 7 nt of the origin were obtained from the randomly primed cDNA library. Therefore, these were accepted as an accurate determination of the ELV 5' terminus.

The 3' termini were determined from viral RNA that was enzymatically poly A tailed. The last non-adenosine nucleotide, determined for these viruses from four or more independent clones, was considered the 3' terminus. The final four nucleotides (GCCC) of PLPV, PeIRSV and ELV are identical to those of several other carmoviruses: CarMV, CCFV, GaMV, SCV, TCV, and PFBV (see section III.F., below), as well as to viruses in other genera of the family *Tombusviridae*: maize chlorotic mottle virus (MCMV), panicum mosaic virus (PMV), and tomato bushy stunt virus (TBSV) (Carrington et al., 1989; Ciuffreda et al., 1998; Guilley et al., 1985; Hillman et al., 1989; Nutter et al., 1989; Skotnicki et al., 1993; Turina et al., 1998; Weng et al., 1997).

Nucleotide sequence and genome organization. The nucleotide sequences for the overlapping clones were assembled using Autoassembler (ABI) and analyzed using GeneJockey II and GCG. The almost complete nucleotide sequence of PLPV contains ~3875 nt, has been deposited with GeneBank (accession number XXXX), and is presented in Figure III.A.2.a. The almost complete nucleotide sequence of PeIRSV contains ~3905 nt, has been deposited with GenBank (accession number XXXX), and is presented in Figure III.A.2.b. The almost complete nucleotide sequence of ELV contains ~3884 nt, has been deposited with GenBank (accession number XXXX), and is shown in Figure III.2.c. The genome organizations predicted from the sequences are shown in Figure III.A.3. For all three viruses, five ORFs were identified. The first ORFs encode a putative protein of about Mr 27,000 (p27). If a readthrough event occurs at the amber termination codon, the ORFs extend to about nt 2333 to produce proteins of about Mr 86,750 (p87). An alternative possibility for these viruses is initiation of translation at an in-frame AUG codon at/near nt ~850, downstream of the amber stop codon. This would yield putative proteins of about Mr 56,122 (p56) (not illustrated).

There are two small overlapping ORFs (ORFs 3,4) located in the central region of the genomes. The first of these ORFs encodes a putative protein of ~Mr 6800 (p7). The next, ORF 4, which is out of frame with ORF 3, encodes a putative p9 protein of ~Mr 9400 with true termination codons. However, the ORF 4 of none of these viruses contains a start AUG codon, an observation validated by performing several sequencing reactions of at least two clones in this region for each virus. This anomaly is addressed in the discussion.

The 3' proximal ORF 5 for these viruses encode the p37 coat proteins (CP) with Mr ~36,552 to ~36,699. These viruses contain 150 to 254 5' end non-coding nucleotides.

In these viruses there is also a single additional ORF larger than Mr 5,000 predicted from the sequences; overlapping, but out of frame with, the end of ORF 5 CP. These ORFs encode a putative protein of ~5400 (p5) Mr protein. However, no nucleotide or amino acid sequence identities were found among these proteins and any sequences deposited in the GenBank databases.

Sequence comparisons. The amino acid sequence identity comparisons between PLPV, PeIRSV and ELV to other carmoviruses, as well as viruses in other genera of *Tombusviridae*, are shown in Table III.A. below.

Table III.A. Percentage amino acid identities between PLPV, PelRSV, ELV and related viruses

Virus	p87			p7			p9			p37CP		
	PLPV	PelRSV	ELV	PLPV	PelRSV	ELV	PLPV	PelRSV	ELV	PLPV	PelRSV	ELV
<i>Carmoviruses</i>												
PLPV	100	57	57	100	57	53	100	46	52	100	49	50
PelRSV	57	100	70.4	57	100	67.2	46	100	67.9	49	100	70.4
ELV	57	70.4	100	53	67.2	100	52	67.9	100	50	70.4	100
PFBV	43	30.0	41.5	34	31.0	32.8	-	-	-	35	32.9	32.6
SCV	38	41.1	39.7	51	32.2	36.2	24	24.4	20.7	34	42.6	33.1
CarMV	38	41.3	41.3	38	33.3	32.8	25	28.0	26.8	26	34.6	34.0
TCV	38	42.0	41.4	33	26.2	26.8	22	21.7	17.0	14	30.2	27.8
CCFV	36	41.1	41.0	33	20.3	22.4	27	29.8	-	19	29.9	27.8
CPMoV	31	37.6	36.5	22	18.8	19.0	24	28.6	20.2	14	23.7	23.1
MNSV	35	38.1	39.0	24	14.3	21.4	16	20.3	15.0	10	23.1	24.0
GaMV	34	36.4	34.0	25	23.4	17.9	21	22.4	20.7	10	22.2	23.7
<i>Tombusvirus</i>												
TBSV	33	32.4	-	-	-	-	-	-	-	18	27.2	26.0
<i>Machlomovirus</i>												
MCMV	33	37.8	-	-	-	-	-	-	-	8	15.1	15.4
<i>Necrovirus</i>												
TNV	38	30.1	-	-	-	-	-	-	-	8	15.1	15.4
<i>Panicovirus</i>												
PMV	37	38.8	-	-	-	-	-	-	-	10	22.8	21.3
<i>Dianthoviruses</i>												
CRSV	27	28.3	-	-	-	-	-	-	-	10	20.1	19.8
RCNMV	28	28.0	-	-	-	-	-	-	-	10	22.8	21.3

- indicates the comparisons were not performed, or no analogous ORF was present.

Additional abbreviations and sources for sequences used: MCMV, maize chlorotic mottle virus (Nutter et al., 1989);

CRSV, carnation ringspot virus (Ryabov et al., 1994); TNV, tobacco necrosis virus (Coutts et al., 1991);

RCNMV, red clover necrotic mosaic virus (Xiong & Lommel, 1989)

3. Discussion

The almost complete 3875, 3905 and 3888 nucleotides of the PLPV, PelRSV and ELV genomes, respectively, have been determined from cDNA clones. Comparing the amino acid identities of putative proteins revealed 18-70% identities, including the p27 protein (not shown). However, *in vitro* expression of this protein was nominal in SCV (Weng et al., 1995). The amber stop codon is believed to be leaky and allow ribosomal read-through of p27 to express the RdRp, a feature common to carmoviruses (Stotnicki et al., 1993; Weng et al., 1997; Ciuffreda et al., 1998).

Based on conventions used for distinguishing strains and species of potyviruses (Shukla and Ward, 1989 a,b), the sequence percentage identities observed between PelRSV and ELV do not support considering them

synonymous. The identities are, however, considerably higher than pair-wise comparisons among other carmoviruses. The ~50% identity between the coat protein amino acid compositions probably reflects several highly conserved and immunogenic sites that are reactive to polyclonal antisera. This would account for the inability to distinguish the viruses in ELISA or western blots.

The readthrough domain of PLPV, PelRSV and ELV p89 is identified as the putative RNA-dependent RNA polymerase as it contains the GDD motif and surrounding conserved amino acid residues characteristic of most viral RNA-dependent RNA polymerases (Kramer & Argos, 1984; Argos, 1988). As shown in Table III.A., the readthrough domains of PLPV, PelRSV and ELV share moderately high amino acid sequence identities with the putative polymerases of the known carmoviruses CarMoV, PFBV, TCV, SCV, CCFV, MNSV, CpMoV, SCV, GaMV, as well as to other members of *Tombusviridae*. It has previously been reported that the polymerase domains of several of these viruses share a high degree of amino acid sequence similarity with each other (Carrington et al., 1989; Nutter et al., 1989; Reviere & Rochon, 1989; Weng et al., 1997).

The predicted genome organization of PLPV, PelRSV and ELV is similar to those reported of carmoviruses, with 5 ORFs, including one coding for a 36-38 Mr CP. There are, however, several important distinctions. PLPV, PelRSV and ELV contain a single subgenomic (sg) RNA species whereas carmoviruses contain two sg RNAs (Morris & Carrington, 1988). The estimated size of the sg RNA (Figure III. A. 3.) suggests the p6/p7, p9, and p37 CP genes are all translated from the single sg RNA. Multicistronic sg RNAs are not typical features for plant virus gene expression, although there are examples of viruses that appear to use this strategy (Rochon & Johnston, 1991).

The general model for arrangement of the p6/p9 genes, as seen in CCFV, SCV, and TCV, is expression as overlapping, but out of frame, genes from the larger sg RNA and expression of the CP from the second smaller sg RNA. This has been experimentally demonstrated for SCV (Weng et al., 1997). MNSV deviates from this arrangement by having two p7 ORFs (7A, 7B) separated by an in frame amber termination codon that may be read-through to produce a p14 fusion protein. MNSV is like other carmoviruses, however, in having two sg RNA species (Riviere & Rochon, 1990).

The arrangement of PLPV p7, PelRSV p6, ELV p7, and PLPV/PelRSV/ELV p9 differs even more because no AUG start codon is present for the p9 of either PLPV, PelRSV nor ELV. Although we can not exclude non-canonical start sites for p9, there is circumstantial evidence that these genes are expressed as a fusion protein by a -1 frameshift mechanism. Such -1 frameshift mechanisms have been demonstrated in the expression of RdRp genes of several plant viruses including, RCNMV, barley yellow dwarf virus, potato leafroll virus, and southern bean mosaic virus (Kujawa et al., 1993; Kim & Lommel, 1994; Futterer & Hohn, 1996). The frameshift involves tRNA slippage around a shifty heptanucleotide site proximal to a stop codon. Although different heptanucleotides can serve as shifty sites, most conform to the generalized sequence X XXY YYZ (triplets represent frame codons; X= A, G, or U; Y= A or U; Z= A, C, or U). The heptanucleotide CAATTTTC is found immediately 5' to the p6/p7 stop codons for PLPV, PelRSV and ELV, but this exact sequence is not found in any of the 'other' carmoviruses. We plan to conduct *in vitro* translation studies from

these viral RNAs and full length clones to experimentally investigate whether PLPV, PeIRSV and ELV p6/7 is expressed by a frameshift mechanism.

By analogy to TCV, these two small proteins are likely viral movement proteins. Wen-zhe et al. (1998) reported that both the p8 and p9, but not the CP, are necessary for TCV cell-to-cell movement in *Arabidopsis*. Also, Turina et al. (1998) suggests expression of the PMV movement protein(s) as a fusion protein by a -1 frameshift mechanism. This seems to indicate a similar situation as we predict for PLPV, PeIRSV and ELV, although it does not necessarily suggest an overall close relationship to PMV (Turina et al., 1998). PMV differs sufficiently, however, from previously described viruses within *Tombusviridae* to warrant placement in a new genus provisionally called *Panicovirus*. Similarly, we believe that if experimental evidence supports our genome organization models, especially with respect to the -1 frameshift mechanism, PLPV, PeIRSV and ELV warrant designation as members of a new genus within the family. Their uniqueness and fundamental difference from carmoviruses is already validated by the transcription of a single sg RNA species. The evidence presented here that these three distinct viruses, PLPV, PeIRSV and ELV, all appear to have this very similar genome organization also supports the description of a new genus.

B. Comparison of serological- and nucleic acid-based technologies for the detection of PLPV in geranium {US National Arboretum, Beltsville}

Several different cultivars of geranium were inoculated with purified PLPV in December 1997. A side-by-side comparison between serology and hybridization with dig-label PLPV probe performed at the same time (5/28/98) by taking leaf tissue, mincing/mixing, and processing 0.3 g of tissue for antigen-coated ELISA and total nucleic acid extraction for dot blots. The PLPV probe is PLPV 16-A (i.e., the 3' probe form section III.A. above). The results of this test are shown in Table III.B.

Table III.B. Detection of PLPV in PLPV-inoculated geraniums using a 3' PLPV digoxigenin-labeled probe or polyclonal antisera

	<u>Dig-labeled probe</u>	<u>ELISA A405</u> <u>anti-PLPV</u>	<u>Symptoms</u>
1. Healthy geranium (mix)	-	-	-
2. Healthy C. quinoa	-	-	-
3. PLPV-infected C. quinoa	+	+	+
4. Geranium 12	+	+	+
5. Geranium 13	+	+	-
6. Geranium 14	+	+	+
7. Geranium 15	±	-	-
8. Geranium 16	-	-	-
9. Geranium 17	±	-	-
10. Geranium 18	-	-	-
11. Geranium 19	+	+	+
12. Geranium 20	+	+	+

As shown in this table, there is general agreement between ELISA and dig-labeled probe hybridization for the detection of PLPV in PLPV virus-inoculated geraniums. There is a possibility that the probe may be slightly more sensitive as suggested by the faint positive probe reaction for geranium samples 15 and 17 which were ELISA negative during the May 1998 test but were then weakly ELISA positive 3 months later.

C. Isolation and characterization of a PLCV isolate from pelargonium {Volcani Center, Bet-Dagan}

1. Methods and Results

Virus isolate. The virus was identified in Israel in *P. zonale*, *P. peltatum* and *P. domesticum*. Symptoms were expressed at the end of winter. Small yellow spots which later became necrotic appeared on young leaves. The leaves became wrinkled and curled. Pelargonium plants grown at high temperature did not express symptoms. The virus was mechanically transmitted from pelargonium leaves showing symptoms to *C. quinoa* and *Nicotiana clevelandii*.

The incidence of PLCV in Israel seems to be very limited and only a very few PLCV-infected plants were found.

Virus purification. Inoculated leaves of *N. clevelandii* 4 days after inoculation were homogenized in two volumes 0.2 M phosphate buffer pH 7.6 containing 0.1% mercaptoethanol. After centrifugation at 10,000 g and filtration through Kimwipes, the homogenate was emulsified with an equal volume of 1:1 butanol-chloroform. After centrifugation, the aqueous phase was layered on 20 ml 20% sucrose cushion and ultracentrifuged in a Beckman 35 rotor at 34,000 rpm for 3 hr. The resulting pellet was resuspended in 1 ml 0.03 M phosphate buffer pH 7 and layered onto a 10-40% sucrose gradient and centrifuged at 27,000 rpm for 2.5 hr. Fractions containing the highest concentration of virus were centrifuged and the resulting pellet was dissolved in 0.03 M phosphate buffer pH 7.

Purified virus was injected subcutaneously into rabbits at two weeks interval. Three injections, each consisting of 0.3-0.5 mg of purified virus, emulsified in an equal volume of Freund's incomplete adjuvant, were administered. A fourth injection was given intramuscularly. Blood was collected periodically, starting one week after the fourth injection. Antisera were evaluated in EM decoration tests and ELISA.

Determination of capsid protein molecular weight. The molecular weight (Mr) of coat protein was determined by SDS-PAGE. Gels 12% polyacrylamide (75 x 5 mm), were prepared as described previously. The protein bands were either stained with 0.1% Coomassie Brilliant Blue or electroblotted to nitrocellulose and probed with the polyclonal antiserum. Antibody binding was detected using anti-rabbit IgG conjugated to alkaline phosphatase (BioMakor, Rehovot, Israel).

Sucrose gradient-purified preparations from *N. clevelandii* contained spherical particles of PLCV of about 28 nm in diameter. The viral coat protein contains one protein of approximately 37.5 kD and a single stranded RNA.

Serology. The antiserum had a titer of 1:10,000 in ELISA when PLCV-infected *N. clevelandii* was tested. It gave good results with PLCV isolate from Germany and medium results with PLCV isolate from Holland. Our isolate gave good results with an antiserum obtained from Denmark and poor results with an antiserum obtained from Italy (Table III.C.). This data suggests a serological difference between geographically different PLCV isolates.

Table III.C. Reactivity of PLCV isolates with different antisera

Isolate	Antibodies to PLCV isolates		
	Israel	Denmark	Italy
Israel	++++	++++	+
Germany	++++	NT	NT
Holland	++	NT	NT

D. Tomato ringspot virus (TomRSV) monoclonal antibodies {US National Arboretum, Beltsville}

Mouse hybridoma cells secreting antibodies to TomRSV were produced as follows. BALB/C mice were injected three times with 50 ug of purified TomRSV with adjuvant over a period of two to three months according to an immunization schedule developed in the lab. One week after the third injection, mice were given a fourth injected with purified TomRSV in PBS. A single cell suspension was prepared from the mice spleens, and cells were fused with FOX-NY myeloma cells four days after the last injection. Antibodies in culture fluids were determined by ELISA using freshly prepared TomRSV-infected cucumber. Seven antibody-secreting cultures were identified, expanded and cryopreserved. These cell lines have not yet been cloned, nor tested as to their utility in the detection of TomRSV in geraniums.

E. Tomato ringspot virus (TomRSV) {Volcani Center, Bet-Dagan}

The virus was intercepted in pelargonium cuttings (*P. zonale* Concolour Lace) imported from Germany. The virus was transferred to indicator plants and tested by serology to confirm virus identity. The virus was transmitted to *C. quinoa*. Tip necrosis were consistent with that reported for TomRSV.

F. Cloning, sequencing and genome organization of PFBV {Volcani Center, Bet-Dagan}

1. Methods.

Virus purification and RNA extraction. PFBV was propagated in *C. quinoa*. Virus purification was carried out as described previously (Franck and Loebenstein, 1994). Viral nucleoproteins of purified PFBV in 0.05 M Na acetate pH 7.2 were dissociated in a solution containing 1% sodium dodecyl sulfate (SDS). After extraction in phenol-chloroform, viral RNA was precipitated with sodium acetate and ethanol (Sambrook et al., 1989) and resuspended in water. RNA was treated with DNAase to eliminate possible DNA

contamination.

RNA isolation and molecular cloning. Viral nucleoproteins of purified PFBV in 0.05 M Na acetate pH 7.2 were dissociated in a solution containing 1% sodium dodecyl sulfate (SDS). After extraction in phenol-chloroform, viral RNA was precipitated with sodium acetate and ethanol (Sambrook et al., 1989) and resuspended in water.

Complementary DNA (cDNA) synthesis was primed with random hexameric oligonucleotides and performed using a commercially available kit (Riboclone, cDNA Synthesis System, Promega). The cDNA was blunt-end ligated into the SmaI restriction site of plasmid pUC19 and used to transform competent *Escherichia coli* JM109 cells (Sambrook et al., 1989). Recombinant colonies were screened by ampicillin resistance with negative-galactosidase activity. The sizes of DNA inserts were estimated by agarose gel electrophoresis following digestion by Bam HI, Hind III, Sac I and Eco RI. The identity of the DNA inserts was determined by Northern hybridization. Purified viral RNA was transferred to nitrocellulose filters and hybridized with 32P-labeled recombinant plasmid DNA generated with random oligoprimers (random primer DNA labeling kit, Boehringer Mannheim). The cDNA inserts in recombinant plasmids were excised and evaluated for size by agarose gel electrophoresis. The largest insert obtained with the Bam HI digestion was gene-cleaned from gel slices and subcloned into Bam HI site of plasmid pGEM-7Zf(+) (Promega), which contains opposing T7 and SP6 RNA polymerase promoters. Plasmids containing the 1500 bp cDNA fragment, designated G1, were linearized with Nsi I and Eco RI for transcription with T7 and SP6 RNA polymerases. Radioactive cRNA transcripts of the G1 clone were obtained using 32P-uridine 5' triphosphate (UTP) as the labeled nucleotide (Melton et al., 1984; Sambrook et al., 1989).

Poly (A) tailing of the PFBV viral RNA. For cloning the 3'-region of PFBV, a poly (A) tail was appended to the viral RNA 3' by Poly (A) polymerase. To a 50- μ l reaction volume the following were added: 40 mM Tris-HCl, pH 8; 10mM MgCl₂; 250 mM NaCl; 250 μ g/ml purified viral RNA; 250 μ M ATP; 50 μ g/ml BSA and 5 units of Poly (A) polymerase and incubated at 37°C for 30 min. The reaction was stopped by adding 2 μ l of 0.5 M EDTA and heated to 75°C for 10 min. After phenol extraction and ethanol precipitation the tailed RNA was collected by centrifugation. The pellet was washed with 70% ethanol and dried briefly.

A c-DNA first strand was reversed transcribed from the poly (A)-tailed RNA and the 3' region was amplified by Taq polymerase reaction by using a specific sense primer near the 3' region and an anchored tagged poly dT primer. The amplified fragment was cloned into T/A pGEM cloning vector and after transformation to *E. coli* JM109 competent cells, white colonies were selected. Plasmid DNA was prepared and sequenced from two separate clones. The results show that a full 3' region of PFBV is contained in these clones.

First strand c-DNA synthesis of the Poly (A)-tailed PFBV RNA. Poly (A)-tailed PFBV RNA pellet was resuspended in a small volume of water and annealed after heating for 10 min at 75°C to the 100 pmoles of Anchor tagged poly d-T primer 5'-ACTAGTGATTGCGGATCCGTGGACGCGCCGCTTTTTTTTTTTT -A/C/G. Reversed transcription was

performed in a reaction volume of 20- μ l following the manufacturer instructions (Boehringer Mannheim).

PCR-amplification of the first strand c-DNA of PFBV 3' end. PCR was performed with 1/10 volume of the cDNA in a reaction volume of 50 μ l. 10 pmoles of each the oligo-dT-dT primer and a specific 3' PFBV primer (5'-CGGACTTCAGGATTAGGT, complementary to nucleotides 2847-2864 of PFBV genome) were used. PCR cycles were performed as the following: 1 cycle 95°C 3 min; 5 cycles: 95°C 30 sec, 50°C for 2 min, 72°C for 2 min; 30 cycles: 95°C 30 sec, 54°C for 2 min, 72°C for 2 min; 1 cycle 72°C for 60 min.

Cloning of 3' end PCR-product of PFBV into T/A pGEM vector. PCR products were separated by agarose gel electrophoresis. A band of 1-kb length was excised out of the gel by GeneClean Kit (BIO101) and ligated to T/A pGEM vector following the manufacturer instructions (Promega, Madison, U.S.A). After transformation of JM109 competent cells, white colonies were picked and plasmid DNA was prepared. Restriction analysis was performed on few clones and those, which were consistent with PFBV known sequence were selected for further sequencing analysis by using the specific PFBV primer (5'-CCCGTATGCTACCTTGAC- 3' complementary to nucleotides 3232-3250 of PFBV genome).

Cloning and sequencing of PFBV 5' end. PFBV specific primer (5'-ACCCATCTCCTTGAAGG 3', complementary to nucleotides 827-842 of the PFBV genome), was used to synthesize the first strand cDNA from PFBV viral RNA as specified for 3' end). Then terminal transferase was used to add a homopolymeric deoxy A-tail to the 3' end of the cDNA as follows: First strand cDNA was phenol extracted and ethanol precipitated. cDNA was collected by centrifugation and washed with 70% Ethanol. Pellet was dried briefly.

Tailing the cDNA with Homopolymer of dA. cDNA pellet was resuspended in a small volume of water and added to the terminal transferase reaction that includes the following: 5 μ l of TdT 10X buffer, 4 μ l of 1mM dATP, 6 μ l 5mM CoCl₂ and 5 units of TdT enzyme (Boehringer Mannheim). Reaction volume was adjusted to 50 μ l with water and incubation made for 15 min at 37°C. Reaction stopped by heating for 10 min at 75°C. Tailed cDNA was phenol extracted, and ethanol precipitated as described.

Amplification and reamplification of the tailed cDNA. First round PCR was carried on the tailed cDNA by using PFBV specific primer (5'-ACCCATCTCCTTGAAGG- 3', complementary to nucleotides 827-842 of PFBV genome) and mixture of three oligo-dT-dT primers: each has 12 consecutive dTs and it incorporates a 17-nt T7 RNA polymerase promotor-derived site (in bold) in addition to anchor with either C, G or A residues: 5'-GTAATACGACTCACTATAGGGC-T(12)-A/C/G. PCR cycles were performed as the following : 1 cycle 95°C 3 min; 5 cycles : 95°C 30 sec, 46°C for 2 min, 72°C for 2 min; 30 cycles: 95°C 30 sec, 50°C for 2 min, 72°C for 2 min; 1 cycle 72°C for 60 min. The amplified 5' region was reamplified by second round of nested PCR with the following primers: PFBV-complementary sense primer (internal to the primer used in cDNA synthesis) (5'-CTCCTGCTGCCAGCTATG, complementary to nucleotides 462-480 of the PFBV genome) and T7 specific primer contained in the oligo-dT (5'-GTAATACGACTCACTATAGGGC). PCR cycles were performed as the following : 1 cycle 95°C 3 min; 5 cycles : 95°C 30 sec, 50°C for 2 min, 72°C for 2 min; 30 cycles: 95°C 30 sec, 54°C for 2 min, 72°C for 2 min; 1 cycle 72°C for 60 min.

Cloning of 5' end PCR-product of PFBV into T/A pGEM vector. PCR products were separated by agarose gel electrophoresis. A band of about 700-bp length was excised out of the gel by GeneClean Kit (BIO101) and ligated to T/A pGEM vector following the manufacturer instructions (Promega – Madison, USA). After transformation to JM109 competent cells, white colonies were picked and plasmid DNA was prepared. Restriction analysis was performed on few clones and those, which were consistent with PFBV known sequence were selected for further sequencing analysis. Results are still not finalized.

Nucleotide sequencing and computer analysis. Clones in pGEM-7Zf and T/A pGEM vectors were sequenced using T7, SP6, T3 universal primers, and PFBV internal specific primers at the Weizmann Institute of Sciences facility (Rehovot, Israel). Nucleotide and amino acid sequences were analyzed using the University of Wisconsin Genetics Computer Group (GCG) and DNAMAN software. PFBV related sequences were initially identified using the FASTA program. Multiple alignment and pair-wise comparisons were carried out by DNAMAN. Homology trees were constructed based on the multiple alignment files. The following viruses (Acronyms, accession number in GenBank) were used for these comparisons: Artichoke mottle crinkle virus (AMCV, X62493), Barley yellow dwarf virus, strain PAV (BYDV-P, D85783), Carnation mottle virus (CarMV, X02986), Cardamine chlorotic fleck virus (CCFV, M25270), Cucumber necrosis virus, (CNV, M25270), Cowpea mottle virus (CPMV, U20976), Cymbidium ringspot virus (CRSV, L18870), Maize chlorotic mottle virus (MCMV, X14736), Melon necrotic spot virus (MNSV, D12536), Olive latent virus (OLV, X85969), Panicum mosaic virus (PMV, U55002), Pelargonium flower break virus, strain D2002 (PFBV-D, AJ003153), Red clover necrotic mosaic virus (RCNMV, J04357), Saguaro cactus virus (SCV, U72332), Tomato bushy stunt virus (TBSV, M21958), Turnip crinkle virus (TCV, M22445), Tobacco necrosis virus, type A (TNV-A, X58455).

In vitro translation. Purified viral RNA was translated in the wheat germ system as described by Salomon et al (1978). In addition, RNA was translated in a rabbit reticulocyte in vitro translation system (Reticulocyte Type I, Boehringer Mannheim). One ug of viral RNA was translated in 25 ul of the reticulocyte lysate. After incubation at 30°C for 1.5 hr, translation was terminated with 2 vols of Laemmli-dissociation buffer (Laemmli, 1970). 35S-labeled in vitro translation products were resolved by electrophoresis in 12% SDS-PAGE and visualized by fluorography.

2. Results and Discussion

Analysis of PFBV capsid protein and viral RNA. A single 35 kDa CP band was observed when purified virions were fractionated by SDS-PAGE. This band gave a specific reaction with PFBV polyclonal antibodies in immunoblots (results not shown). Viral RNA from purified PFBV resolved into a single band of approximately 4 kb in 1.5% agarose gel. A riboprobe complementary to the 3' end of the viral RNA hybridize to the RNA.

In vitro translation products from PFBV RNA. RNA from virions of PFBV was translated in the wheat germ and the rabbit reticulocyte lysate systems. Translation products directed by PFBV RNA templates were analyzed by SDS-PAGE in 12% gels. In both systems, PFBV RNA seems to direct the synthesis of three

defined polypeptide chains. The major product synthesized is a 35 kDa polypeptide which co-migrates with the major PFBV coat protein and is reactive with antiserum made to the whole virus in western blots. In addition two other products, 45 kDa and 95 kDa polypeptides, were synthesized in both systems (results not shown). These results indicate that major ORFs predicted in the PFBV genome are translated from PFBV virion RNA in vitro.

Nucleotide sequence and genome organization. Sequences obtained from various cDNA clones and terminal clones were compiled and assembled with the GCG ASSEMBLE program. The assembled 3911 nucleotide sequence of PFBV has been deposited in the GenBank database (Accession number #####). The PFBV genome organization predicted from the genome sequence is summarized in Fig. III.F.1. Five major ORFs were identified. 5'-UTR unknown (sequence lacking from 5' proximal end). ORF1, partial sequence begins at nucleotide (nt) 3 and ends at nt 752, encodes for 249 amino acids with a calculated molecular mass of 27,755 Da. ORF2 complete begins at nt 813 and ends at nt 2291, encodes for 492 amino acids with a calculated molecular mass of 56,255 Da. ORF3 is partially overlapping with ORF2, begins at nt 2276 and ends at nt 2482, encodes for 68 amino acids with a calculated molecular mass of 7,036 Da. ORF4 is partially overlapping with ORF3 and in-frame of ORF2, begins at nt 2316 and ends at nt 2636. It encodes for 106 amino acids with a calculated molecular mass of 12,166 Da. ORF5 begins at nt 2637 and ends at nt 3675. It encodes for 345 amino acids with a calculated molecular mass of 36,798 Da. The 3'-UTR begins at nt 3676 and ends at nt 3911. ORF1 ends with a readthrough stop codon at nt 750-752 which is in frame with ORF2. A second readthrough stop codon exists at the end of ORF2 (nt 2289) and is in-frame with ORF4. Major predicted proteins directed by PFBV genome were translated from PFBV virion RNA in vitro. Similar readthrough proteins are present in the replicase genes of CarMV (Guilley et al., 1985; Carrington and Morris, 1986), TCV (Carrington et al., 1989), MNSV (Riviere and Rochon, 1990), CCFV (Skotnicki et al., 1993) and SCV (Weng and Xiong, 1997). A small protein of 8kD encoded by another frame seems to be similar to the p7 protein of CarMV.

The PFBV genome organization is very similar to that of CarMV. ORF1 in both viruses is in frame readthrough with ORF2 and the latter is in frame with ORF4 by a second readthrough. ORF3 overlaps ORF4 with few nucleotides. ORF5 presides next to ORF4 and does not overlap with any of the ORFs.

Determination of terminal sequences. The terminal nucleotides of the 3' proximal region and the 5' terminus was determined from cDNA clones obtained by traditional cloning and modern techniques that allowed precise determination of the terminal nucleotides. Terminal regions were cloned by the anchored oligo-dT-dT and PCR amplification with a specific internal PFBV primer from poly(A) tail appended to the genomic RNA (see materials and methods).

Comparison of PFBV nucleotide sequence with related viruses. The almost complete 3911 nucleotides of PFBV genome, except the exact 5' terminus, have been determined from cDNA. The sequence significantly aligns to several published sequences of Carmoviruses (Table III.F.). Among Carmoviruses, CarMV and SCV are the most closely related to PFBV with a nucleotide identity of 55.2% and 53.8%, respectively. Moreover, the 7 nucleotide sequence 5'-CCCGCCC-3' is identical at the 3' end of the three

viruses. Studies aimed at elucidating the 3' structure of Tombusviruses strongly suggest the importance of the GCCC sequence for stability of the progeny RNA after transcription and moreover, replication was found to begin at /or downstream of the G residue at position -4 from the terminus (Dalmay et al., 1993). Such features support the close relationship of PFBV with CarMV as a type member of Carmoviruses.

Table III. F. Percentage nucleotide amino acid identities between PFBV and related viruses

Virus	Nucleotide Identity (%)	Amino acid identity(%)					
		ORF1	ORF2	ORF3	ORF4	ORF5	Fused 1,2,4
CarMV	55.2	37.2	65.7	47.2	46.2	38.3	54.3
SCV	53.8	44.6	58.4	44.1	46.1	44.5	47.5
CCFV	47.9	32.3	47.7	32.9	35.4	28.9	35.0
TCV	35.1	27.7	43.1	32.5	-	21.8	36.9
TNV-A	47.5	5.7	43.1	29.6	-	14.3	30.5
MNSV	46.6	22.0	49.6	26.4	44.3	21.7	35.4
CPMoV	45.8	20.5	48.5	26.0	-	21.7	27.4
TNV-D	44.6	5.7	40.0	20.8	-	14.3	30.5
MCMV	44.1	11.6	14.1	20.2	-	13.9	9.3
PMV	43.6	15.6	38.1	20.3	-	13.0	26.7
OLV	42.6	18.1	41.5	28.8	-	15.0	30.1
CRSV	42.2	15.6	32.3	11.9	-	21.4	18.6
TBSV	42.1	13.2	37.0	16.3	-	22.1	25.7
TBSV-D	41.7	12.4	31.8	16.3	-	22.1	25.3
RCNMV	41.5	16.0	33.0	7.4	-	19.1	25.0
AMCV	41.5	12.7	32.0	16.3	-	21.9	24.8
CNV	41.4	12.6	37.5	16.3	-	20.1	25.9
BYDV	40.5	5.7	29.7	11.3	-	12.5	21.4

(- Indicates no significant sequence alignment); AMCV, artichoke mottle crinkle virus (Tavazza et al., 1994); BYDV-PAV, barley yellow dwarf virus (Miller et al., 1988); CCFV, cardamine chlorotic fleck carmovirus (Skotnicki et al., 1993); CNV, cucumber necrosis virus (Rochon and Tremaine, 1989); CPMoV, cowpea mottle virus (You et al., 1995); CRSV, cymbidium ringspot virus (Dalmay et al., 1993); MNSV, melon necrotic spot virus (Riviere and Rochon, 1990); MCMV, maize chlorotic mottle virus (Nutter et al., 1989); OLV, olive latent virus; PMV, panicum mosaic virus; TBSV and TBSV-D, tomato bushy stunt virus (Hearne et al., 1991); RCNMV, red clover necrotic mosaic virus (Xiong and Lommel, 1989); SCV, saguaro cactus virus (Weng and Xiong, 1997); TCV, turnip crinkle virus (Carrington et al., 1989); TNV-A and D, tobacco necrosis virus strain A (Meulewaeter et al., 1990) and strain D (Coutts et al., 1991).

Comparison of genome organization in Carmoviruses. The predicted genome organization of PFBV is most similar to the previously reported one for CarMV (Guilley et al., 1985). However there are several distinctions when PFBV and CarMV are compared to SCV (Weng and Xiong, 1997). In CarMV, a double readthrough event that was predicted to extend the translation of p87 (analogous to fusion of ORFs 1 and 2 of PFBV) into the in-frame of p9 (analogous to ORF4 of PFBV) resulting in a fusion protein of 98000 Mr (Guilley et al., 1985). A protein with approximate size was indeed observed in an in-vitro translation reaction of CarMV when supplemented with suppressor tRNA (Harbison et al., 1985) and PFBV. This double

readthrough event is not predicted in the SCV genome. Although, the p86 ORF is in-frame with p9, but several additional terminator codons between the p86 amber terminator and the p9 initiation codon preclude the second readthrough. Moreover, in-vitro translation data for SCV also do not support a double-readthrough event. In the case of PFBV such additional terminator codons do not exist between the fusion of ORFs 1, 2 and the initiation codon of ORF4. Therefore, it is suggested that the genome organization of PFBV is most similar to CarMV, based on sequence homology and the in-vitro translation products of the genome. Several factors have been suggested to promote ribosome bypassing the first AUG initiation codon. They include short 5' noncoding leader sequences, absence of considerable secondary structure downstream of the 5'-proximal AUG codon to the first initiation codon. Further studies are needed to address the mechanism of gene expression for PFBV.

Comparison of the predicted amino acid sequences of PFBV. Proteins involved in virus replication: Functional genetic studies allowed the identification of proteins needed for Tombusvirus replication. Evidence stemming from these studies suggests that proteins encoded by the two 5' proximal ORFs (ORFs 1 and 2 of PFBV) appear to be the only products necessary for virus multiplication in infected protoplasts (Russo et al., 1994). The amino acid sequence encoded by ORF1 does not contain distinct motifs that may help us to understand its role. However it was shown that the function of this ORF is necessary for the replication of Tombusviruses (Dalmay et al., 1993). The RNA polymerase motif Gly-Asp-Asp (GDD) (Kramer and Agros, 1984) which is located in the readthrough portion of the 92 K protein of Tombusviruses exists in the fusion of ORFs 1, 2 and 4 of PFBV. This may imply a potential role of the proteins encoded by ORFs 1, 2 and 4 of PFBV in virus replication. Additional motifs like the most conserved amino acid motif for NTP-binding proteins (Gly-Xaa-Gly-Lys-Ser/Thr), is lacking in PFBV. This motif is also lacking in other Tombusviruses and Carmoviruses (Russo et al., 1994). Functional genetic studies are needed to ascertain the predicted functions of these ORFs in the replication of PFBV.

Proteins involved in virus movement: Mutational analysis of two overlapping ORFs in the middle of TCV genome has indicated that two small proteins p8 and p9 are involved in virus cell-to-cell movement (Hacker et al., 1992). Homologous proteins (p6 and p9) were expressed from genomic RNA of SCV by in-vitro translation (Weng and Xiong, 1997). Proteins encoded by ORFs 3 and 4 (in the middle) of the PFBV genome show a high degree of identity with the amino acid sequence of the homologous p6 and p9 of SCV, and p8 and p9 of TCV (45% and 46%, respectively; Table III.F.). No significant identity was found with any protein from other viruses. It is expected that variability in the Carmovirus movement proteins, which may reflect the diverse host ranges of each Carmovirus, will stem from the variances within the sequences of homologous p6 and p9 carmoviruses proteins.

Capsid protein: Amino acid sequence comparisons of ORF 5 encoding a polypeptide of 35 kDa which might function as a CP of PFBV shares less identity than amino acid sequences encoded by ORFs 1, 2, 3 and 4 when aligned with homologous proteins from other Carmoviruses. PFBV CP is only 34% and 46% identical to CaMV and SCV, respectively. As with the polymerase protein, CP shares similarities with several other small isometric viruses. Conversely, several other small isometric viruses have significant similarity with

PFBV replicase, but less similarity with CP (Table III.F.).

IV. Evaluation of the research achievements, description of the cooperation and overall conclusions

The focus of this proposal was to further characterize some of the viruses infecting pelargonium and develop better methods for their detection and control.

The biological, biochemical and serological properties of four isometric viruses - PLPV, PelRSV, and PFBV isolated from geraniums exhibiting line and ring pattern or flower break symptoms, as well as an isolate of elderberry latent virus (ELV; which the literature indicates is the same as PelRSV) have been determined. All four of these viruses have been almost completely sequenced and their genome organizations determined. This work has shown that ELV is, in fact, a distinct virus; albeit serologically related to PelRSV. All of these four viruses should each be considered new, distinct members of the Carmovirus group of the family Tombusviridae. Hybridization assays using recombinant DNA probes also demonstrated that PLPV, PelRSV, and ELV produces only one subgenomic RNA in infected plants. This unusual property of the gene expression of these three viruses suggests that they are unique among the Carmoviruses.

The development of new technologies for the detection of these viruses in geranium was also demonstrated. Serological reagents (polyclonal and monoclonal antibodies), nucleic acid hybridization probes to PFBV (radioactively-labeled cRNA riboprobes) and to PLPV (non-radioactive digoxigenin-labeled cDNAs), and RT-PCR technologies have been developed and/or refined to detect at least two of the 'pelargonium' viruses directly in geranium. Hybridization probes developed were generally shown to be no more sensitive for the detection of virus in infected plants than the standard ELISA serology-based assays. However, the RT-PCR was shown to be over 1000 times more sensitive in detecting PFBV in leaf extracts of infected geranium than was ELISA.

All these achievements were within the aims of the original proposal and should lead now to a better understanding of the identity of the viruses infecting pelargonium and to the development and utilization of new tools that can be used in an improved scheme of providing virus-indexed pelargonium plants. The sequence information, and the serological and cloned DNA probes generated from this work, will allow the application of these new tools for virus detection, which will be useful in domestic and international indexing programs which are essential for the production of virus-free germplasm both for domestic markets and the international exchange of plant material.

The proposal was jointly planned and prepared. Reports and ideas were exchanged by e-mail and personal meetings. Cloned cDNAs of PLPV, PelRSV and PFBV, and sequence data and antisera against the different viruses were exchanged. Dr. Gera and Dr. Frank spent two weeks in Dr. Jordan's Lab. The project was interactive in many respects, but especially in exchanging materials and joint work. Four MSs were published and at least 3 more (two joint) publications are in draft stages and almost ready for submission to journals and we hope to continue this cooperation.

V. List of publications to date from this project:

Franck, A., Gera, A., Antignus, Y., and Loebenstein, G. (1996). Detection of pelargonium flower break carmovirus using ELISA and a transcribed RNA probe. *Acta Horticulturae* 432:338-344.

Franck, A., Loebenstein, G., and Gera, A. (1997). Use of the reverse transcription-polymerase chain reaction for the detection of pelargonium flower break carmovirus. *J. Phytopathology* 145:235-238.

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VII. Figures cited in Section III

Figure III. A. 1. Northern hybridization analysis of PelRSV RNA.

Fig. III.A.1. Northern hybridization analyses of PelRSV RNA. Blot is also representative of analyses of PLPV and ELV RNAs. (A) Hybridization of 3' (left panel) and 5' (right panel) dig labelled PelRSV probes to PelRSV RNA (1), dsRNA extracted from *C. quinoa* (2), and total nucleic acid extracts from PelRSV infected *C. quinoa* (3). (B) Hybridization of 3' (left panel) and 5' (right panel) dig labelled ELV probes to ELV RNA (1), dsRNA extracted from *C. quinoa* (2), and total nucleic acid extracts from ELV infected *C. quinoa* (3). Standard is digoxigenin labelled RNA marker (Boehringer Mannheim).

Figure III. A. 2. a. Nucleotide sequence of PLPV.

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1  CTCGCATCTT  GGGGGAGGCA  CTGCAATTGG  GTGTGTTTCG  CGTCAAGGCC  AGTTGTTGGG
61  TCAGTAAGGA  GTTGGCGCTC  CTTGGTCCCA  ACCTTGCCCTG  GATAGCCGCC  AGACCACTTC
121  GCAACGATGC  TAATGCCATT  CTAGAGCGAA  TTGGCGTCCA  CGGGTTGGCC  AAAATCCCGT
181  GCAACTTCGA  CCCCAGATGAG  CTGACGATCG  CAAATGACTG  TATTGTGAAG  TCCGACAGGA
241  ATCTGGAGGA  TGAATCAGCA  GTGGTGGAGG  AGATCGATGA  GGGGGAGGAG  AAAAAAGAAG
301  GCAAGAAGGT  GACAAGGAGG  AGGGTGAAGA  CCAAGCCTAC  CTTGCGGCC  GTTCTGGCGG
361  CTGACGCGAA  GAACTATTAC  GGTGCTCTCC  CGAGTGCCAC  CAGGGCAAAC  GAGCTCAGCG
421  TCATGAAGTA  CTTAGTCTCG  AAGTGTCGAA  AGCACAAACT  GACAATAACT  CAGACACGAG
481  AGGTTAGTGC  CATGGCTTTT  GCGTTAACCT  TCACGCCAGA  TGAGAATGAT  AAACCTCATCC
541  ACAAAATATCT  TAATTCAACC  GAGGTTTTTT  AACGCCGGGT  GGATTATGCC  AAGGCGCGTG
601  GGGTCGACAA  GTGCTGGTTC  GAATTGCTCA  AGAGGCCCTG  GCACGCGCGC  GCATGGAGGA
661  GAGTGGTTGG  GCGGATTTTC  GGAATGCCTG  AGCAGCAGGC  TTTGAGTTTC  GTTAAATAGG
721  GATGCCTCAT  GGACACTTGT  GGTGTGGACA  CGAAAGTGTG  CCGCGGGGAA  CACCACTGGG
781  TTAAGGAGTT  CCGTGGGGCG  GCAAACCCCA  AGCCGCGTAG  GTTGTATAAG  ATTAGTGGTG
841  TATCCCTGTA  AGTTAGATGG  GGGGTGCACA  ATAATAGTTT  TGTCAACCTC  AGAAGAGGGT
901  TGATGGAAAAG  GGTTTTCTAC  GTCGAAAGAG  GTGGGGAGCT  CCTACCATGC  CCATCTCCGG
961  AGGCAGGTTT  GTTCAAACGG  TTGTTCAACA  AGGTTGGACG  GCGGGTGATT  AGGTTTTGCG
1021  GCCATCATTC  CCCGATCCCC  AGGGCTTCTT  ATCCTGGGAT  GTTTCAGGGC  AGGAAACGCA
1081  CGATCTATGA  AAACGCTGTT  CGGTCCCTTG  TTGACCGACC  TTATAATATT  AGAGATAGTT
1141  ATTTAAAGAC  TTTTGTAAAG  TTTGAGAAAT  TAGACTTTAG  CAAGAAACCT  GATCCCGCGC
1201  CTAGGGTAAT  CCAACCCAGG  CATCCGAGGT  ACAACGTAGA  ACTAGGAAGA  TATCTGAAGC
1261  CTTTGTAGCA  TTTTGTGCTAT  AAGGCACTAG  ACAAGTTGTG  GGGAGGGCCC  ACCGTTATGA
1321  AAGGATACAC  CGTTGAGGAA  ATGGGAGGGA  TTATCAAGGA  CAGCTGGCTC  CAATTCCAGA
1381  AACCAGTGGC  TATTGGGTTC  GACATGAGTC  GATTCGATCA  ACATGTTTCT  GTGGATGCGC
1441  TGAAGTTTGA  GCACAGCATT  TACAAGGCCT  GCTTCTCGAA  GGATGGGAAT  CTGGCGACGT
1501  TGCTTGGTCA  CCAGATATCT  AATAGAGGCT  CTGCTTATGC  AAATGATGGC  TACTTGAGAT
1561  ATAGAGTGGA  AGGGAAAAGG  ATGAGCGGGG  ACGTAAACAC  AGGGCTGGGG  AATTGCTTGT
1621  TGGCGTGCAC  TATCACTAAA  TTTTAAATGG  AAGAAATCGG  CGTGAGGTCC  AGACTGGTGA
1681  ACAACGGGGA  CGACTGCGTG  CTTATATGTG  AAGCCGGCGA  TTGCCGCGCT  GTCGAGGATA
1741  GCTTGACAGT  GGGGTGGCGA  CGGTTTGGCT  TCACCTGCAT  TGCGGAAAAA  CCCGCTACG
1801  AACTGGAAAA  GATCGAATTT  TGCCAGATGA  GCCCTATACA  GATCAGTGAT  TCTCGCGTCA
1861  AACTAGTGAG  AAAGCCCCAG  AAAAGCATAT  CCAAAGATGC  ACATTCCACC  ACCCACTCA
1921  CCACCATACA  GCTAGCTCAA  GAATGGACCC  GCGCTATCGG  AGAAGGCGGG  TTATCTCTCA
1981  CCAGTGGCAT  CCCAGTAGTC  CAGGAATTC  ACCAATGCCT  CATCCGTAAC  GGAAAGAAAA
2041  CGACCAGGGA  AACAAAGAAA  AGGGCCTTTT  ACGGGGACTA  CTATTGGAAG  TGGGTTAACC
2101  AGCATCACGG  CAAGTATGAA  CCGGTGACTG  AGGCAGCTCG  GCACTCATTC  CACTTGGCGT
2161  TTGGGGTTAG  CCCTGACCAA  CAGTTGGCTC  TAGAGGCCAT  ATACTCTCGC  AAGTCGTTAC
2221  TTTGGGAGTC  CTCCCCCTTT  ACATTTGAAG  TTGGCGCTAT  CGAATGGATA  TTCAACCAAA
2281  AGATACCAAT  CTGAGTGTA  AGGAAGCAGG  GAAATCAGCA  AAGCGCGGAA  GCACAAAGAA
2341  CAAGCTCGAC  GTAGCTCACA  GTGGAGTGTC  CAAGGGCACA  TCTCGCGATC  TTGTCGGTGC
2401  TAATTTTGTC  ACAATTGCTG  ATAAAGTGGA  ATTTGTGGTC  CATCTCAATT  TCTGACTTAC
2461  TACCACAGCC  ACATTTGTTG  CACCCCAATC  TACTTGTCTG  CATCGTGTG  TGCATCTTCT
2521  TTAGTTCAGT  TCTTAGCCAG  GGACAGAGTT  ATAGTTATAG  TTATATTTCC  ACCAGCACTA
2581  GCGATAAATT  CATCTCAGTA  GCTGTAGGAA  ATGGCGGCCA  AGGATAATCC  GGAGTTCATC
2641  GCAGCCGTGG  CAAGACGGGA  GCAGTGGGCA  ATTAAGCTTC  AATCCAAAGG  TTGGGGCTCT

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2701 TTGTCCAAAG CCCAGAAAGC CACTGCACGC TCGTACGGGA TAGGGAACCC TCCAACAGTC
 2761 GTGGTGCCTC GCACCACTCG GCTGGTGGCA GGGAAACCCA CGAACGCACG GCGTGC GCGG
 2821 GGGGAGCCTG GTAATGCCAA GATGGGCACC ACCATAACCA AGCAGGAATA CATGGGCGAG
 2881 CTTCAATTCTT CTTCAAAGAT TGAAACTTAC TTGCTCGACC CCAAGAATGT GAGGAGCTTC
 2941 CCTCAGTTAA GCGGGCTTGC AATCGGCTAC AACAAGTACA AGATCACTGA CTTCAAGGTG
 3001 CGGTACAGTC CTAAATGCAG CGACAGTGGG TGTGGGTTGA CCATTGCCTA CTCTAGCGAC
 3061 TCATCTGATC CACCGCCGAA GAACAAATTC AACTTGTTTA GCATGAACCA CAAGTATGAA
 3121 ACAGCCGCGC ACAAGCCACT CCTAGTAACA CTTCCCGTAT CTAAAGAGAC CAGATTCTTG
 3181 CGGGATAAGT GCACTGATGA TTCCAAGCTC GTAGACAGTG GGGCGGTGCA TGTCCTGGTG
 3241 GACGGGAACC ACGAAGGTAG GCTCGGAGAA CTTTTCTTCG AGTTAACTAT CGTGTTCTCT
 3301 GAACCAACCT TTTGCAATCA CCACTCCCAA GTCATTAAGG GAATCAAGGA GAGTGAAGGG
 3361 CCCGAATACG TCAAAGTTGA GCCTGACAAG GAAACCGTGA AACTAGTTTT CCAGGCAGCG
 3421 GGGGCATACT TAGTGTCTAT AGTAGCCTCC AAAGTAGAGC GAATCGGGCA GCTCGGTGCT
 3481 TCTGAAGGTG ACAAGCAGGT AGTAGAGAGT GCTGATAAGA GTGCCTGCCT CTGTAGGATA
 3541 GAGGCGGAGC AACCAGGTAG TGCTATAGTA TTAGTTTATG CCAGCAGGGA GGCCATTACC
 3601 CTGCGAGCTT ACATCAACAA GCTATGAGGA AAGCCACTGC GGTGGGGTCA GTCTCCTGGT
 3661 GCGCCAACAC CCAGAAGGGG AGACAACTTC AGTGGTTCCA AACCCGACGC TCAATGTAAG
 3721 CCTTGAAAAA TCCAAAAACA GAGTGCTACT GCTTTCAGTG TGCGAGCTTT ATTTGCTCAC
 3781 TTCCTCCGTC GATAAAATCC CGAATTTTCG GGTGGGCTTC GGCGGACATC CTTAATGAAG
 3841 AACGGGGGGG TAACCCACCC CCCTGATCTG CCC

Figure III. A. 2. b. Nucleotide sequence of PelRSV.

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1 NTCTCAACTC GCGTTATGAC TGACGTTGTT AAGTTTGGGG TTTGGGCRGG GTTTTACCTG
61 GCCAAGGAGT TGGCGCTCCT TGGACCAGAG TTGGCCAGCA TTACACTTTC TAGCTTGA CT
121 CCGAATTATA ATGGATTGTT GACCTCCATT GGA CTGCCAA CCGTTAGTTC CATACCGACA
181 GCATCCCCCC CTATAGATTT GGTTC TAGTG AACGACGGTG TACTCAACAC ACCTAACCTA
241 GAGTTGGAGG GTGAGCTTGT GCCACTCTTG GAGGAAGTGA TAACTGATCC GAAAACCGGA
301 ACGATAACAA AGGCAGTGAA AACTCGGCGA CCAAAGAGCA AGACCATTTC CTCTTG CATA
361 CTAGCTGCAG AGACCAAAAA CCATTTTGGC GGGCTTCCTA GGGCGACAAG GGCAAACGAG
421 TTAAGCGTGA TGAAACATCT GGTCAACAGG TGTAAAGAAT GCAAGTTGAC TGCCCTCCAA
481 ACTCGGGAGG TTTCAGCCAA AGCTTTTTC C TCGTGTTCA CCCCCGACTC ACATGACAAG
541 TTCATCTACG AATTCTTAAA CAGTGACATA ACGTTTGAAC GTAGGTGTGA CTACTTAAAA
601 AGCCAGAGGG TGGATTCCCTG TTGGTTGAGA CTGTTGCAGA ATCCCTTTTG GAAGAAAAGG
661 TGG AAGGCAG TTGTTTGTAG GCTTATGGGC ATGGGCGTCC AGGAGGCCTA CGAGTTTGT
721 AAATAGGGGT GCCTCTCTAG CACACCCGGT GTGGATACAA AGATCTTCAG GGGGTCCAC
781 CGGTGGGTTT GTGAAGTTAG AGAGGATGTT CCCCAGTAC CTAGGACGTT GTACAGAGTA
841 GAAGGCCTCA GTGATGACCA ACAATGGGGC ACTCACAATA GCAGTTTCCT AAAC TTGAGG
901 AGGGGGTTGA TGGAGAGGGT ATACTTCGTC GAAAGGCAAG GAACACTCAC TCATTGCCCC
961 CCCCACAAG CTGGGGTTTA TAAAAGGTTG TTTACAAAGG TTGGCCACAA GTTGGTG GGA
1021 TTGTGCGGGA ATCATTC CCC GATCCCCAGG GACCAGTACC CTGGGATGTT TCAGGGCAGG
1081 AAGAAGACAA TTTACACCAA CGCAGTGGCC TCTCTTTCCG ACATGCGGTA TAATGTTAGA
1141 GATAGTTACT TGACAAC TTT TGTGAAATGT GAGAAGATTA ACTTTAGCAA GAAGCCTGAT
1201 CCAGCTCCTC GGGTGATACA GCCACGCACT CCACGGTACA ATGTTGAACT TGGCGTGTAT
1261 CTCAAGCCCG TAGAGCACAG CATATACAGA GCGATTGACA AGGTGTCGGG AGAACCAACC
1321 GTGATGAAGG GCTACACTGT AGGAGAGATG GGGAAAATCG TTGAGGACAA GTGGAATAAG
1381 TACCTGGCAC CTGCAGCGAT AGGTTTTGAC ATGTCTAGAT TTGATCAGCA TGTGTCAGTG
1441 GACGCTTTGA AGTATGAACA CAAAATCTAC CGGAAAATTT TTTCGAACGA CACGCACTTA
1501 AGGCTTTTGC TTGAGCACC AATAACCAAT CAGGGCGTGG CGTACGCTAG GGATGGTAGG
1561 TTGCATTACA AGGTCGTGGG GAAGCGCATG AGTGGGGACG TGAACACATC ACTCGGAAAC
1621 GTGATTCTTG CTTGCTCCAT AAGCAGATTG CTAATTAAAG AGTTGGGGGT GAAGGCATCT
1681 TTGATCAACA ATGGGGACGA TTGCGTGCTA ATAGGTGAAC TCAAAGACAT CAGGAAGGTG
1741 GAGGGTATCT TGGCCGATTA CTGGTTGGGA TTTGGGTTCA CTTGCATTAG CGAGGCAATA
1801 GTGACAACAT TAGAAAAGAT TGAATTTTGC CAGATGTCGC CAATATATAT AGGCAACGCT
1861 TACAAAATGG TCAGGAATCC GCGGACGAGC CTATCTAAAG ATGCCCATAG CACTACGCCC
1921 TTTGAAACTG TGAAAGTGGC GAGGGAGTGG TGCCACTCAG TGGGGCTTGG TGGACTCGCA
1981 CTTACATCTG GCGTCCCAGT TGTCCAAGAA TTCTACAAGT GTATGATACG TCACGGAGTG
2041 GAGCCAAGGA GGGACAAAAT GAGACACTTT TGGGGGGAGT ACTGGTTGGA GTTTGTTAAA
2101 ATGCACAAGG GGGGGGAGCA GCCAGTATCT GAGGAAGCTC GGCACAGTTT CCACTTGGCG
2161 TTTGACATTT CTCCTGACCA ACAACTTGCC CTGGAAGGAT ACTACAAATC CCTTGAGCTA
2221 AACATGGAGT TCTCCCGGCC AAGTAACAAT TTGGCGGTTG TTGCAGACAT TGAATGGATA
2281 CTCAGAAACC CAGTGGCAGT CTAGCAAACA AAGAAGTTGT GAGAGGCAAA CCCAAGAAAC
2341 TCGACGTAGC GCATTCTGGC ATCTCCAAAG GTACCAATTC AAGTACTACT GGTGCTGTTT
2401 TCGTTACTGT GGCTGAAAAG GTGGAGTTTG TCGTTACTCT CAATTTCTAA ACCATCATTT
2461 GACAACCCGA GCTTGTCAT ATCTATTGTG CTCGGTCTGT TCTTCGCATC TGTATTAACA
2521 CCAGGCAATT ATAGTTATTA TTATTATTCC GCTAATTCCA CAAGTGACAA GTTCATCTCT
2581 GTAGCTGTGG GAAATGGCCA GCAATAGTCC CATAGTACTG GCGGCGGCTA ACCGGGGTGA
2641 AGTTTGGGCG GTCAAGCTGA AGCAATCTGG GTGGAAGACG CTTAGCAAGG CTCAGAAGGC

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2701 AGCTGCTCGT GCTCAAGGCA TAGGAGCTGC CCCATTGAGC CAGACTGTGC CGAAGACCAC
 2761 CAGGATTATA CAAGGAAACC CAACCTTCTC CCGTCGATCC CGAGATGCTC CCGGGAACGC
 2821 TCAGAAATCC ATGACCATCG TGAAGTCTGA GTTTCTTGGA GAGGTTTCGG CAGATGGGGC
 2881 AATCCACACT TACACACTTG ATCCCCGCAA CGTTCGAACC TTTCCCCATA TCTCCGACCT
 2941 GGCYCGTGGG TTCAACAAGT ACAAGTTTGG GGACTTGAAA GTACGATATA GCGCCAAGGT
 3001 CCAGGARTCT GGCTGTGGCG TTTGTATTGC TTTCAACCCG GATTCATCTG ACCCCAAGCC
 3061 GAAAGGTAAA TTCGATTTAT ACTCACTTGG AAGTAGGGCT GAAGGGGCCG CTCATCGAAA
 3121 CCTGCTATAC ACAGTCCCAA TTGACAAATC TCAAAGATTG TTGAGAGACT GTGCGACCGA
 3181 AAATTCAAAA GACGTGGACG CTGGTAGTTT GTTTGTGTTG GTGGATGGCC AGCACGATGG
 3241 GCGAGTCGGG GAACTCTTCC TCGAAGTGAC CATTGTGTTG AGCCAGCCCA CGTACAACCA
 3301 GCGGGCCACA CAACTTTTGG CAGGTACCAC ACACAGAGAC GGACCGACTT TTGTGAAGGC
 3361 AGTGAAAAGC GCTAATACCG TCTCGTTAAC TTTCCAGGCA GCTGGTAACT ACCTGGTTAG
 3421 CACGTATAGC TCTCTGCTGG AACGGGTTGG TCGGCTCGCG TTGGGCGAGG CAGAACAATC
 3481 ACAGGTGGAT TCGTCCACAC ACAGTAGCAA TGTAGTAGAA GCTCTAGTTG CCGCACCTGG
 3541 CGGAAGTATT GTATATTACT TTAAGACCCC TGATCAGATG TCGTTCAGGG CTTATGTTTG
 3601 CCGTATGTGA ATGGTGGGAT GGGGGGGTTT TGGTAATCAT TAGGGCTACA GGACAACCGA
 3661 GGAGGCGCCA ACCTTCGACC ATCGGTGATC CCCTATTGCA CTCCAGCCAC CCATTGTTAC
 3721 CTTCACTAGG CACCACTTAA GAAAAATCCA AAAACAGAGT GTTAGGAGTA GGCATAGCTA
 3781 CACCGGAACC TAAAATCCCT TCGGGGTGGG CTGGAACCGG GCCTGTAATG AATTGGGGGG
 3841 AGGGTTCCCA TCCCCTTTAA TGCCC

Figure III. A. 2. c. Nucleotide sequence of ELV.

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1  CGCTTGACTA  GTCAC TAAGG  GAAAA CACCC  CCATCTACAG  CTATGGGACT  GATCACACAA
61  TGCATCTGGT  TTGGAGCTTA  CGTTGGGTTT  AAGGTTACCA  AGGAGTTGGC  GCTCCTTGGG
121  CCGGAAC TCA  CAAGTATCAC  ACTGTCCTTG  GGGTTTCCTG  AAATCAACCC  AGTGCTAGCA
181  GCCATAGGGC  TGCCCACCGT  ARACTACATC  CCTGAAGTTA  ATCAGGTTGA  CGTGTGTGAT
241  GCCACGAATG  ACGCCATTCT  GCACACCCCC  GACGTCGAAT  TAGAGACTGA  GCTCGAGGAC
301  TCTAGGGTCC  TCACCATTGC  TGAACCGGAG  ACCCCCCGGG  TAACCACCGT  GGTGAAAAC T
361  AGACGGCCAA  AGAACAGGCG  CACTTTCGCT  TGCCTCTTGG  CAGCGGAAGC  AAAGAACCCAC
421  TTCGGGGGGA  TGCCCAGGAA  CTCTCGGGCT  AACGAGCTCA  GCGTCATGAA  GTATCTTGTG
481  GGGAAAGTGCC  AGGATCACAA  GTTGACCGCC  CTCCAGACTA  GGGAGGTCGC  GGCCAGGCG
541  TTTGCACTGA  CGTTCACCCC  TGACGCCAAT  GACAAGTTCA  TATACACCTT  CCTTAATTTC
601  GAAGACGCTT  ACGATCGAAG  GTGTGACTAT  GTCAAGAGTC  AGGGAGTGGG  CCCGATGTGG
661  CTGAGGTTGT  TGAAGCGGCC  TTTTAGCCGA  AAGGAGTGGG  AGCAGGTGGT  GTGTAAGTTC
721  ATGGGTTTTG  GGACCCGTGA  GGCCTTACAC  TTTATTAAAT  AGGGGTGCCT  ATCGCAATCC
781  CCCGGTGTGG  ACACAAAGAT  TGAGAGGGGC  ATGCACCGGT  GGGTTAGGGA  AATGCGGTGG
841  GAAGGGACGC  CCAATCCTAG  ATCACTGTTT  AGGTTAGAGG  GCCTCAGCGA  CAACCAATGT
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961  TTCGTTGAGC  GTAACGGCCA  ACTAGTGGAC  TGTCTCAAC  CGCTTCCCGG  GCGTGTGCG
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1141  GTTGCTAGCT  TGAATGATGT  GCCTTATAAC  ATTAGAGATA  GTTATTTAAA  AACCTTTGTG
1201  AAATGCGAGA  AAATTAAC TT  CTCGAAGAAA  CCGGATCCTG  CTCCCAGGGT  AATTCAGCCT
1261  CGGTCCCCAC  GCTACAATGT  AGAGTTGGGC  CGCTACCTTA  AACCTGTGGA  GCATRGCGTG
1321  TACAGAGCGA  TTGATAAGTT  GTGGGGGGAA  CCCACCGTCA  TGAAGGGATA  CACTGTAGAT
1381  GAGATGGGAG  AGATCGTTAG  GACTAAATGG  GAGAAATACC  AGAAGCCTGC  TGCTTTAGGA
1441  TTCGATATGT  CGCGATTTGA  TCAGCACGTT  TCTGTGGACG  CCCTGAAGTT  CGAGCACAAA
1501  ATTTACCGGC  GCATGTTTAG  TGGGGATGAG  AACTTAGCTT  TACTGCTGAG  CCATCAAAATC
1561  CACAAAAGG  GCGTCGCGTA  TGCTAAAGAT  GGCAGATTGA  GTTACAAGGT  TGAGGGTAAA
1621  CGGATGAGTG  GGGATGTGAA  CACATCTCTT  GGAAATGTCA  TCCTCGCGTG  TTCCATTAGC
1681  CGCTACATAC  TGGGCGGATT  GAAGATTAAG  GCTTCGCTGA  TCAACAATGG  GGACGATTGT
1741  GTGCTGATAG  GTGATTTGAG  GGATATCAGG  CGGGTGGAGG  GTATCTTGGC  CCAAGCTTGG
1801  TTGGA CTTCG  GATTTACTTG  CATTAGCGAA  CCAATCGTCA  CCACTTTGGA  GAAAATCGAG
1861  TTTTGT CAGA  TGTCCCCAGT  TTTGGTCGGT  GAGGGATACA  AGATGGTGCG  GAACCCGAGA
1921  ACTAGCTTAT  CCAAGGATTG  CCATAGCACC  ACCCCATT TG  AGAACACTCG  GGTGGCTGCT
1981  GAATGGGCCT  ACTCAGTCGG  GCTGGGCGGA  CTGGCGCTAA  CCTCAGGCGT  CCCTGTTGTC
2041  CAAGAATTCT  ACAGTTGTCT  CCTACGTAAC  GGAATGAAGC  CTAAGAAAGA  CAAGATGGAC
2101  CACTTCTGGG  GTGAGTACTG  GATGGAGTAT  GTGAGAATGC  ACAATGGGAA  GCATGAGCCG
2161  GTGACTCAAG  AGTCCCGGCA  CAGCTTTCAT  TTGGCCTTCG  ACATTTCTCC  GGATCAGCAA
2221  CTTGCTCTGG  AAGAATACTA  CCGGTCCGTC  GACTTGCTTA  TGGAGTTCGC  CCCCAGAAGC
2281  GACTACTTGG  CGGTGGTCGC  ATGGATACTA  GAGCACAAGA  TAAGGATATA  GACCAAGGGA
2341  AAGCAGCTGC  ATTGAGGAAA  ATAGAACGAG  GCAAACCTAA  GAAGCTTGAC  GTAGCACACT
2401  CTGGCATCAG  CAAGGGAACA  CATTCTGACA  CTACTGGTGC  TGTGTTTGTC  ACTGTTGCTG
2461  ATCAAGTCAC  ATTTACGTTT  ACCCTCAATT  TCTAGACCTT  CCTTCGACAG  CCCAAATCTC
2521  TTAGTCTCCA  TTGTTCTAGG  ACTATTTTTC  GCTTCTGTTT  TAACACCCGG  TAATTACGTG
2581  TATAGTTATT  ACTCTGCAAA  CTCAACGAGT  GACAAGTACA  TCTCGGTTGC  TGTAGGAAAT
2641  GGCCAATAAA  AGTCCAGTCG  TGGTTGCAGC  GGCCGGGCGC  GGTGAGCTGT  GGCAGTGAA

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2701 ACTGTCGAGT TTGGGGTGGC GGAGTCTGAG CAAAGCCCAA AAGGCCAGCG CTCGGGCGGC
2761 GGGCATTGGG GCTGTGCCTC AGTCCGCCAT TATTCCCCGC ACAACTCGGT TAATAGCTGG
2821 CAACCCTACG AACTCTCGCC GCACCCGGGA CGCTCCTGGC AACGCGCAGA AGAGCATGAC
2881 TGTCTTCAAG TCGGAGTTCT TAGGTGAGCT CCATGCGGAC AATACCATTG GGTCTTACAA
2941 GCTAGACCCC CGCAACGTCC GCACCTTCCC GCACATATCC GACCTCGCAA GAGGCTTTAA
3001 CAAGTACAAA TTCCTGATG TGAAGGTTAG GTACAGCTCG AAGGTGCAGG ACTCTGAGTG
3061 TGGGCTCACG ATCGCTTTCT CCGCTGATTC CAGCGACCCA GCACCTCGCG GCAAGTTTGA
3121 CTTGTACGCC CTGGGGAGCA AGGTGGAGGG CGCGGCCCAC AAAAATCTCC TTTACACTGT
3181 CCCGTTGAC AAAGGACAGC GCTTCCTGCG TGACGGTGAG GGAGACAACG CCAAGGAGGT
3241 GGATGCTGGC GTAATTTATG TGCTGATGGA TGGGGCACAC GACACCCGCG CGGGGGAGTT
3301 ATTCCTCGAG GTGACTGTTG TGCTGTCCCA GCCAACATAC AACCAGCGAT CCACGCAATA
3361 CATCAGCGGC ACAGAGCACC GCGGCGGACC CAACTACGTT AACATCACCA AGAGTGCGAA
3421 CACTATGACG CTTACGTTCC AGGCGGCTGG GACGTTTCTG CTTTGTAGCT ATTCCTCTCC
3481 ACTGGAGAAA GTTGGGCGCC TATCCTTGGC GGATGCGGAC CAATCACAGG TCGACTCTGC
3541 GAATAATAGC AGTAATATAA TAGAGGCTGT AGTAACCACC CCTGGTGGTA GTATTGTATA
3601 TTAATTCAAG TCCCGGGAAC AGATGTCATT CCGGGCTTAC GTTGGACGCA TGTGAACTT
3661 TTTTGCATGC TTGTGGTGAA CAGGACAGCC GAGGGGCGCC AACCCCGATT GTCGGCGAAC
3721 CCACAAGCGG CATGGATGAG AACGTGCCCC GCGTAATAGA AAAATACAAA AACAGAGTGT
3781 GTACATTCTT TCTGAGTTCT CGTCGN (x)

Figure III. A. 3. Schematic of genome organization and gene expression strategies for PLPV, PelRSV, and ELV.

Fig. III. A.2. Schematic of the genome organizations and gene expression strategies for ELV, PelRSV, and PLPV. Each open rectangle represents an ORF and is labelled according to the size of the putative protein each encodes. Shaded box represents putative frameshift of p7.

Figure III. F. 1. Nucleotide sequence of PFBV.

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1   CCTCGGTATC TGGTTCCAAC ATGCTACGAT TCGGATCTCA GTTAGTGGTG
51  GGTTCCACCT TAATCGGTGG TCTCACTGCT GTCGGGCTGG CAGGCCTGAG
101 CGTGCGTGCT ACAATAGGTG CAGTGGAATT CAACCTCCGC TGTGCTAGAG
151 TGGTGGGTGA CATCATACGT GACCCATTTA ATGCCCTCCC TTCGGCCCCCT
201 CTACTACCTG GGCCAGCGT TGAGGTTTTT GCTGAGGAGG TGAGGAAGGA
251 CCTCGAGCCG GAGCTTGAGG CTAAGGATTA CCTTGTCATC CACGAGGAAA
301 AAGAAATGGA TGAGGAGGGA AAGAGCAGGG TGGTTAGGCA GAGGTCTACT
351 GTCAACCGCC ACAAGAAGGG CCGGTTTGTA CACCGGCTAG TCTGTGATGG
401 GAAAAACCACT TTTGGTGGGA CCCCTTCAGC TAGCCGGGCC AACGAGCTGG
451 CTGTAATGAA GTATCTCGTG GGTAAGTGCC GCGAGACCA CTTAGTGGTG
501 CAGCACACCC GCGAAGTCTG CTCTCTAGCG ATGGCTGCCA TTTTCACCCC
551 GGACCACCAT GAAGTAAACA TGGTTAGGGA GATGAACAGT CACGCCGCCT
601 ACCGAAGAAG GGTAGCTCTT GCAGATGCCG CCAGAGTCCA TAGCTGGCAG
651 CAGGAGTTAC TCCGCTCACC CCTGAGTTGG AACGCTTGGG GGAGGGCGTG
701 GTGGATAGCC AACGGGCTCC CTGACAGGGA GCCTGTTCCG TTCACCAAAT
751 AGGGGGGTCT TTTCTGCCTC GAGGGTGTG AAACAGGAGT CCGTAGGGGG
801 ACGCACCCAC AGATGATCGA GGTAGAGAAA AACTCCCCC CGAAACTTAG
851 GAAACTCTTC GCCAACGAT TGGTTAGCAG TGGAGTTGAA TACCGCGTGC
901 ATAACCACTC GTTTCAAAAC CTAAGGCGAG GTCTGCTTGA GAGAGTTTTT
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2901 CAACGTATCG AGCACAACCG GGGTGACCCC GACTTTCCAA TCATGGATCA

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3051 CTGTTCCGAC ACTATGAATG GGAAGGTGGC CATTGGCTTC ACTCCTGATT
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3151 TATGCCGACA CTCCAGCAAA GGAGTCGGTC ACCTTGAAGA CGGCTGCGGA
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3251 TGGATTATGG CCGCGTAGTT TTAGCCACAT ACGGTTTCGA TAGCACAGCT
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3501 GGTGATCAAC TCTCCGGTTA TATCCGGGTC CGGGGCAAGC GGATCCGTAA
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3901 TTAACCCGCC C

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