THE COMPLETE NUCLEOTIDE SEQUENCE OF PRUNE DWARF ILARVIRUS RNA1 AND VIRUS DETECTION BY REVERSE TRANSCRIPTION PCR AND TRIPLE-ANTIBODY SANDWICH ELISA

by

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Department of PLANT SCIENCE

The University of British Columbia
Vancouver, Canada

Date 1/10/96
ABSTRACT

A triple antibody sandwich enzyme-linked immunosorbent assay (TAS-ELISA) with a monoclonal antibody was developed and evaluated for the detection of prune dwarf ilarvirus (PDV) in sweet cherry trees (Prunus avium). A reverse transcribed polymerase chain reaction test was also developed to establish the incidence of PDV in 40 sweet cherry trees and to confirm the absence of virus in 15 control trees. Trees with two-thirds of their leaves positive for PDV by TAS-ELISA would be identified with 99% probability by testing four leaves per tree. The monoclonal antibody did not cross-react with Prunus necrotic ringspot ilarvirus in the TAS-ELISA.

The nucleotide sequence of PDV RNA1 was determined. The RNA consists of 3374 nucleotides and encodes a single open reading frame of 3168 nucleotides. The putative translation product is 1055 amino acids in length with a calculated molecular mass of 118.9 kDa. Both the nucleic acid and the translated amino acid sequences show stronger homology to RNA1 and the corresponding translation product (ORF1) of alfalfa mosaic alfamovirus (AMV) than to citrus leaf rugose ilarvirus, the only other ilarvirus for which RNA1 sequence data is available. There is extensive sequence homology in the 3'-untranslated regions of PDV RNA1 and the 3'-regions of other ilarvirus and AMV RNAs. The reported sequence and its single open reading frame conform to the genomic organization
typical of the *Bromoviridae* genus. Clones representing sequence from the 5'-' and 3'-end of RNA1 were used to construct a deletion-type defective interfering particle and its ability to replicate *in vivo* was assessed.
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<td>AMV</td>
<td>alfalfa mosaic alfamovirus</td>
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<td>ApMV</td>
<td>apple mosaic ilarvirus</td>
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<tr>
<td>AP</td>
<td>alkaline phosphatase</td>
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<tr>
<td>βME</td>
<td>beta mercaptoethanol</td>
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<tr>
<td>BMV</td>
<td>brome mosaic bromovirus</td>
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<tr>
<td>bp</td>
<td>base pairs</td>
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<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CiLRV</td>
<td>citrus leaf rugose ilarvirus</td>
</tr>
<tr>
<td>CIP</td>
<td>calf intestinal phosphatase</td>
</tr>
<tr>
<td>CMV</td>
<td>cucumber mosaic cucumovirus</td>
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<tr>
<td>DAS-ELISA</td>
<td>double antibody sandwich ELISA</td>
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<tr>
<td>DI</td>
<td>defective interfering</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagle medium</td>
</tr>
<tr>
<td>DNase</td>
<td>deoxyribonuclease</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
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<tr>
<td>dsRNA</td>
<td>double stranded RNA</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetra-acetic acid</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<tr>
<td>FCS</td>
<td>foetal calf serum</td>
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<tr>
<td>HAP</td>
<td>hydroxylapatite</td>
</tr>
<tr>
<td>HAT</td>
<td>hypoxanthine aminopterin thymidine supplement</td>
</tr>
<tr>
<td>IgY</td>
<td>hen egg yolk antibodies</td>
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<tr>
<td>IPTG</td>
<td>isopropyl-β-D-thiogalactoside</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
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<tr>
<td>kb</td>
<td>kilobase</td>
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<tr>
<td>MEA</td>
<td>2-mercato ethylamine</td>
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<tr>
<td>Me-HgOH</td>
<td>methylmercuric hydroxide</td>
</tr>
<tr>
<td>NC</td>
<td>nitrocellulose</td>
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<tr>
<td>ORF</td>
<td>open reading frame</td>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
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<tr>
<td>PDA-3C</td>
<td>a murine anti-PDV monoclonal IgG, antibody</td>
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<tr>
<td>PDA-3C-AP</td>
<td>alkaline phosphatase conjugate of PDA-3C</td>
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<tr>
<td>PDV</td>
<td>prune dwarf ilarvirus</td>
</tr>
<tr>
<td>PNRSV</td>
<td>Prunus necrotic ringspot ilarvirus</td>
</tr>
<tr>
<td>Pr</td>
<td>Probability of detection of PDV</td>
</tr>
<tr>
<td>RACE</td>
<td>rapid amplification of cDNA ends</td>
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<tr>
<td>RBDV</td>
<td>raspberry bushy dwarf idaeovirus</td>
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<tr>
<td>RdRp</td>
<td>RNA-dependent RNA polymerase</td>
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<tr>
<td>RNase</td>
<td>ribonuclease</td>
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<tr>
<td>RT-PCR</td>
<td>reverse transcription PCR</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SSC</td>
<td>standard saline citrate</td>
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<tr>
<td>TAS-ELISA</td>
<td>triple antibody sandwich ELISA</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TDT</td>
<td>terminal deoxynucleotidyl transferase</td>
</tr>
<tr>
<td>TCS</td>
<td>tissue culture supernatant</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>TSV</td>
<td>tobacco streak ilarvirus</td>
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<tr>
<td>UTR</td>
<td>untranslated region</td>
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<tr>
<td>X-gal</td>
<td>5-bromo-4-chloro-3-indolyl-β-D-galactoside</td>
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Some of the results in section 3.3.1 to 3.4.2 have been published in the following article:


Dr. K. Eastwell's role in this publication was in helping to plan the overall experiment and in giving advice and criticism during its execution. Dr. J. Hall also gave advice during the planning of the experiment, but was involved principally in carrying out the statistical analysis of the results. My involvement was in doing all of the laboratory work, including the production of the monoclonal antibody, the field indexing by TAS-ELISA, the bioassay and the RT-PCR analysis and in assisting with the statistical analysis.

Results of the nucleotide sequence of RNA1 (sections 3.7.0 and 3.7.1) have been submitted under the title:

Dr Eastwell’s role in this publication was in giving advice and suggestions throughout the duration of the cloning and sequencing. My involvement was in carrying out the cloning, sequencing and the analysis of the sequence.

The sequence of RNA1 has been deposited with the GenBank and assigned the accession number U57648.

(C. Rampitsch)

(K.C. Eastwell)

(J. Hall)
1.0 INTRODUCTION

1.1 Diseases and spread of prune dwarf virus

The ilarvirus diseases of cherry trees are caused by prune dwarf ilarvirus (PDV) and Prunus necrotic ringspot virus (PNRSV). Strains and combinations of these ilarviruses are responsible for at least 11 diseases in sweet cherry (Prunus avium L.) and sour cherry (P. cerasus L.) (Mink & Jones 1996). This review lists seven diseases on cherry cultivars caused by (PNRSV) and six caused by (PDV) with two apparently caused by both. Németh (1986) lists a further two in peach (P. persica L.), three in apricot (P. armeniaca L.), six in plum (P. domestica L.) and two in almond (P. dulcis L.). However, some of these are caused by co-infection of PDV and PNRSV, and one by co-infection of PNRSV with apple chlorotic leafspot trichovirus (Németh 1986). Such dual infections usually cause more severe symptoms than single infections.

Fruit trees infected with PDV or PNRSV show two phases of symptom expression (Gilmer et al. 1975). An acute, shock symptom appears in the first season after infection, or if the infection occurred early in spring, shock symptoms may appear in the same season. Typically these are seen as leaf discolouration, necrotic lesions and may include fruit damage. The second phase occurs in the next season and will reappear annually. These chronic symptoms may be quite mild,
even absent in some cultivars. Chronic symptoms are different from acute and may include any or all of several distinct symptoms including chlorotic lesions, decreased tree-size, reduced percentage of bud-take, delayed ripening and usually a reduced yield. Yield can be reduced by 50% or more in sour cherry yellows disease and this is one of the most serious diseases of sour cherry in North America (Davidson & George 1965). Although PDV does not affect fruit quality, it is a problematic virus for fruit growers since Prunus are propagated vegetatively and the presence of PDV greatly reduces the percentage of bud-take (Gilmer et al. 1975).

I larviruses are spread in three ways. All are spread readily by grafting infected tissue, some can be spread through seed (Casper 1977) and a few have been shown to be pollen transmissible. Western flower thrips (Frankliniella spp.) are likely vectors for the pollen transmissible ilarviruses (Greber et al. 1991; Sdoode & Teakle 1993). With these three modes of transmission, PDV and PNRSV can easily be introduced and spread rapidly through orchards causing losses in fruit yield within a few years (Uyemoto et al. 1992). The most frequent source of new infections in orchards is the introduction of infected material, since fruit trees are vegetatively propagated. It is very important that all bud-wood and seedlings distributed by nurseries are certified virus-free. Seedlings may be
infected if a maternal tree was pollinated with infected pollen (Casper 1977). It is thought that PDV and PNRSV will not infect the maternal tree during fertilization and it remains virus-free unless there is physical damage at the site where infected pollen lands. By analogy to virus transmission to herbaceous hosts, feeding behaviour by thrips appears to provide sufficient damage to allow the virus to infect the tree (Greber et al. 1992).

It has still not been shown that thrips are vectors for PDV and PNRSV in Prunus spp. but based on experiments with herbaceous plants, the circumstantial evidence is overwhelming. Western flower thrips (Frankliniella occidentalis and Thrips madronii) were implicated in raspberry bushy dwarf virus (RBDV, a pollen transmitted idaeovirus) transmission in raspberries (Bulger et al. 1991) and Microcephalothrips abdominalis spread tobacco streak ilarvirus (TSV) through tobacco fields in Queensland, Australia (Greber et al. 1991). More importantly F. occidentalis are able to infect cucumber cotyledons dusted with PDV and PNRSV infected pollen (Greber et al. 1992). The experiments indicated that virus was transferred from the pollen to the plant during feeding presumably entering the plant through cells damaged by the feeding insects. Plants dusted with pollen alone, or caged with thrips alone failed to develop symptoms. The authors stressed that it was unlikely that the thrips were responsible for transporting the infected pollen from tree to
tree, because they clean themselves carefully before flying. This transport role has been assigned to honey bees which pollinate the trees in spring (Uyemoto et al. 1992). Greber et al. (1991) also stressed the importance of weed populations in tobacco fields acting as a reservoir and alternate host for thrips. Further evidence, using enzyme-linked immunosorbent assays (ELISA), Digiaro et al. (1992) found PNRSV and PDV both on the surface and within pollen grains although they could not detect apple mosaic ilarvirus (ApMV) in association with pollen. Kryczynski et al. (1992) have noted a correlation between flowering intensity and the spread of PNRSV through sour cherry experimental orchards.

1.2 Detection of prune dwarf virus

Traditionally, fruit trees are tested for PDV and PNRSV in the spring, by budding onto an indicator tree. The most commonly used indicator for these ilarviruses is P. serrulata cv. Shirofugen (Gilmer et al. 1975). This tree responds to ilarvirus infection by a hypersensitive gumming response at the bud union within four to six weeks when budded with infected wood. Mechanical transmission to cucumber (Cucumis sativus) and squash (Cucurbita maxima) is also possible with both PDV and PNRSV. Both viruses show local chlorotic lesions on the inoculated cotyledons accompanied by a systemic infection. PNRSV infections often result in apical necrosis. Some squash cultivars, such as 'Buttercup', show
chlorotic vein clearing of the true leaves when infected with PDV and this cultivar is frequently used as a source of PDV for purification. PNRSV will elicit ringspot symptoms in Chenopodium quinoa and this plant is sometimes used to distinguish between the two ilarviruses in a bioassay.

Serological techniques have been applied to indexing plant viruses (Clarke & Adams 1977). The most popular is the ELISA because of its flexibility and scale-up potential. It is quicker than the bioassay, yielding results within days rather than weeks and it is very reliable and less expensive, but it does require the availability of antiserum against the virus to be tested. Serological assays are also more specific than bioassays and are able to differentiate between PDV and PNRSV. The reliability of the assay depends to a large degree on the serological diversity of the virus in question and on the ability of the available serum to recognize these serotypes. Polyclonal and monoclonal antisera can both be used in ELISA. In general monoclonal antibodies offer a lower background, but their higher specificity may cause a failure to react with certain virus serotypes. Polyclonal antibodies are generally better suited for viruses with many serotypes, especially if false negative results are highly undesirable. Both mono- and polyclonal antisera specific for PDV and PNRSV are available (Torrance & Dolby 1984; McMorran & Cameron 1983), and have been used in serological assays to assess the extent of virus
infections in Washington (Mink 1984) and California (Uyemoto et al. 1989; 1992) by double-antibody sandwich (DAS)-ELISA.

Perhaps the biggest drawback to serological techniques applied to indexing is this problem of serotypes of viruses which the antibodies may not recognize or recognize poorly. PNRSV is serologically diverse and is serologically related to ApMV, but no distinct subgroups of PDV have been found based on serology (Torrance & Dolby 1984; McMorran & Cameron 1983). The problem of serological diversity can be overcome by using a pool of antisera, if available, or by resorting to alternate techniques such as bioassays, nucleic acid hybridization or polymerase chain reaction (PCR). In a recent survey Crosslin et al. (1992) used radioactively labelled PNRSV riboprobes to confirm ELISA results, since the latter does not detect the CH30 serotype of PNRSV. This serotype was readily detected by nucleic acid hybridization.

PCR and related assays such as reverse transcription PCR (RT-PCR) and immuno-capture PCR, are the most sensitive techniques currently available for the routine detection of plant viruses. Wetzel et al. (1991) were able to detect 10 fg of plum pox potyvirus RNA (approximately 2000 virus particles) in a field indexing trial by RT-PCR. They found this technique to be more sensitive than nucleic acid hybridization with ³²P labelled probes, which had previously been the most sensitive assay for plant virus detection in vivo. Another study comparing monoclonal antibodies, DNA
probes and PCR to detect grapevine yellows disease caused by a phytoplasma, also found that PCR could detect approximately 10 fg of target and was the most sensitive of the three techniques tested (Chen et al. 1993). Similar results were obtained from a study with grapevine fanleaf nepovirus detection in grapevine (Rowhani et al. 1993) where again the authors were able to detect virus in the fg range.

In addition these studies highlight some of the problems encountered with PCR of woody tissue samples (leaves, roots, bark and shoots) because of inhibitory substances present in the tissue. There have been no large-scale PCR indexing studies involving ilarviruses to date. Efforts are now being made to simplify and modify the basic PCR procedure, especially in tissue preparation and detection, so that the technique can be used to index large numbers of plants as rapidly and inexpensively as possible without sacrificing sensitivity (eg. Korschineck et al. 1991).

1.3 Genetics of prune dwarf virus

PDV is a member of the Bromoviridae (Rybicki 1995). This genus includes four genera of plant viruses: the bromoviruses, ilarviruses, cucumoviruses and alfamoviruses. All members are tripartite with icosahedral or quasi-isometric particles. Their genomic RNAs are positive-sense, single-stranded and each RNA is encapsidated separately. The genomic RNAs are 3.2-3.6 kb (RNA1), 2.6-3.0 kb (RNA2), 2.1-
The genome organization of the bromovirus, cucumovirus and alfamovirus genera is well established since several members have been sequenced completely (e.g. AMV: Cornelissen et al. 1983a; 1983b; Barker et al. 1983; Brederode et al. 1980). The genome organization of all Bromoviridae studied to date is identical with analogous genes encoded by analogous segments. RNA1 and RNA2 are monocistronic, encoding proteins thought to be involved in replication since RNA1 and RNA2 can replicate independently of RNA3 in protoplasts (Kiberstis et al. 1981; Nassuth et al. 1981). RNA3 is bicistronic encoding the movement and coat proteins. The latter is translated from the subgenomic RNA. These proteins have been produced in cell-free translation systems (Dougherty & Hiebert 1985); the movement protein (ORF3a) is required for systemic infection of AMV in tobacco leaves (Huisman et al. 1986).

The RNA species of all known Bromoviridae have a 5'-(m7G)5'ppp5'Gp cap but no 3'-poly(A) tail. In spite of these similarities at the genome level, there are important differences in structure, serology and mode of transmission amongst the genera of the Bromoviridae.

TSV is the type member of the ilarvirus genus and the first member for which sequence data was reported. Its RNA3 has been sequenced completely (Cornelissen et al. 1984), and the 180 3'- and 140 3'- nucleotides of RNA1 and RNA2 respectively, have also been sequenced (Koper-Zwarthoff & Bol
The genome organization of TSV has been inferred from cell-free translation assays and is typical of the Bromoviridae organization as outlined above: RNA1 and RNA2 encode 120 kDa and 100 kDa proteins, respectively. These RNAs exhibit sequence homology with RNA replicases from other plant and animal viruses as well as phage (Kamer & Argos 1984). RNA3 of TSV is bicistronic, encoding a 34 kDa movement protein and a 24 kDa coat protein translated from a subgenomic RNA4 (van Tol & van Vloten-Doting 1979). The following ilarvirus sequences are also known: ApMV RNA3 (Alrefai et al. 1994; Sanchez-Navarro & Pallas 1994), PNRSV RNA3 (Guo et al. 1995; Hammond & Crosslin 1995), PDV RNA3 (Bachman et al. 1994), citrus leaf rugose virus (CiLRV) RNA3 and citrus variegation virus RNA3 (Scott & Ge 1995b), lilac ring mottle RNA3 (Scott & Ge 1995a) CiLRV RNA2 (Ge & Scott 1994) and CiLRV RNA1 (Scott & Ge 1995c). All of these sequences are consistent with the Bromoviridae genome organization typified by AMV. There are at least two known exceptions to this organization: Di Terlizzi et al. (1992) reported an extra subgenomic RNA of unknown origin associated with PNRSV and Ding et al. (1994) showed that an extra subgenomic RNA in cucumber mosaic cucumovirus (CMV) preparations is derived from RNA2 and contains an ORF of 100 codons. The function of the potential gene product, ORF 2b, is not known and it is not known whether either of these RNAs are translated in vivo.
The ilarviruses and AMV require all three genomic RNAs plus either a copy of the coat protein or the subgenomic RNA4 to initiate infection. The coat protein has been shown to bind structural hairpins at the 3’-ends of the RNAs (Houwing & Jaspars 1982) and it is thought that the coat protein of AMV is located in the replicase complex. The 3’-untranslated region (UTR) of all known ilarvirus sequences (and of AMV) shares these structural hairpin motifs which are flanked by AUGC. Coat protein activation is not specific and the coat protein of TSV can provide some early functions for the replication of AMV even though the two share little sequence homology (Reusken et al. 1995). BMV and CMV do not require coat protein to initiate infection.

Species of the bromo- and cucumovirus group have also been sequenced and their genome organization is the same as that of AMV. However, the 3’-UTRs fold into a tRNA-like structure and can be charged with tyrosine in vitro (Hall et al. 1972). The function of this aminoacylation is not known but there are some theories. The 3’-end of these viral RNAs has other tRNA-like functions: when aminoacylated they are able to interact with GTP and a host elongation factor, and if supplied with ATP can repair incomplete or broken 3’ viral CCA termini (the site of aminoacylation of host tRNA). Analogy to tRNA is structural only and there is almost no sequence homology with tRNA (Perret et al. 1989). Deletion analysis of the 3’-region of BMV RNA and subsequent
replication in vitro suggests that the tRNA-like structure has a promoter function, but this in vitro replication system has not been perfected and these findings are tentative (Dreher & Hall 1988). It is not known whether BMV RNA is aminoacylated in vivo; replication of BMV RNA proceeds in the absence of free tyrosine in vitro. Finally it has been suggested that aminoacylation of the 3'-end of viral RNA is a strategy to protect against exonuclease degradation.

1.4 Defective Interfering RNA

Defective interfering (DI) particles are RNA species derived from the supporting virus and can not replicate in its absence. DI RNAs may be either deletion-type, mosaic-type or snapback-type (Schlesinger 1988). Deletion DIs generally contain the 5'- and 3'- termini of the supporting virus genome which are essential for replication, but have deletions in the open reading frame, which yields no functional product. Mosaic DIs share sequence with non-contiguous portions of the supporting virus genome and have the 5'- and 3'- termini of the supporting virus genome. There is no functional gene product. The snapback type DIs are long palindromes which can form either hairpins or panhandles and do not encode a functional gene product. Both termini are derived from one terminus of the supporting virus genome. DI particles are believed to arise by the copy-choice mechanism of the RNA-dependent RNA polymerase (RdRp)
in a scheme proposed by Lazzarini et al. (1981).

Although DI particles are quite common in animal viruses, they rarely arise from plant viruses (eg. Finnen & Rochon 1993; Hillman et al. 1987) and there are only two reports of a natural DI particle in Bromoviridae (Romero et al. 1993; Marsh et al. 1991). However, artificial DI particles from two Bromoviridae, BMV and beet necrotic yellow vein cucumovirus and from cymbidium ringspot tombusvirus replicate in vivo and attenuate disease symptoms (Hehn et al. 1994; Marsh et al. 1991; Kollar et al. 1993). These DI particles are of the deletion-type. Attenuation of disease symptoms and reduction in the titre of the supporting virus are frequently caused by DI particles although there are rare cases where symptoms are exacerbated by DI particles (Romero et al. 1993). Because of this potential symptom attenuation, because they are unable to replicate in healthy trees in the absence of helper virus and because they can be synthesized in vitro from cDNA clones, DI particles have been investigated for controlling plant diseases (Hull & Davies 1991).

1.5 The nucleotide sequence of PDV RNA1

The complete nucleotide sequences of several members of the Bromoviridae have been determined (section 1.3). Among the ilarviruses, only CiLRV has been sequenced completely (Scott & Ge 1995b; 1995c; Ge & Scott 1994), and the sequence
of AMV is also known (see section 1.3). Since all RNA viruses (except retroviruses), irrespective of their host, must encode an RdRp and since this gene is highly conserved, it is used extensively in phylogenetic analysis among unrelated viruses (Kamer & Argos 1984). The RNA1 segment of the Bromoviridae encodes part of the viral replicase complex, and this RNA, along with RNA2 (which also encodes a replicase protein), should provide useful information to establish the relationship of PDV with other Bromoviridae and with other ilar- and ilar-like viruses such as AMV, olive latent virus 2 (OLV2, an alfamo-like virus, Grieco et al. 1990) and RBDV.

Phylogenetic analyses of the ilarviruses and studies of their genetic relationships to other Bromoviridae have been made possible as the RNA3 sequences of eight ilarviruses have recently been determined (section 1.3). The ilarviruses have been divided into 10 sub-groups based on serological assays. These groupings are similar to phylogenetic groupings observed in some studies using RNA3 sequence, where a close genetic relationship has been observed between PNRSV and ApMV (subgroup III: Guo et al. 1995) and between CiLRV and citrus variegation virus (subgroup II: Scott & Ge 1995b). However, not all ilarvirus RNA3 segments have been sequenced and the phylogenetic analyses presented in these studies are not complete and may be inaccurate. Sequences of ilarvirus RNA1 and RNA2 segments are even scarcer and are also required to form a complete phylogeny of the ilarviruses. Closely
related viruses such as AMV and OLV2, will have to be included in a final phylogenetic analysis.

1.6 Objectives of this study

In order to control a plant disease a reliable detection method for the causal agent is essential. The first objective was to produce a TAS-ELISA assay based on a monoclonal antibody and to establish a routine diagnostic procedure, determining the reliability and accuracy of the serological assay to detect PDV in infected material.

At the beginning of this study, there was no sequence data available for PDV and thus there were no means of developing an RT-PCR assay for the detection of PDV. The second objective was to obtain sequence data from RNA3 of PDV to produce a reliable RT-PCR test. The RT-PCR would be used to evaluate the TAS-ELISA results.

The third objective was to obtain the complete sequence of RNA1 of PDV. Analogy to other members of this family suggest that RNA1 would encode a putative replicase enzyme. This sequence information would be used to construct artificial DI- particles to be used as potential biocontrol agents for PDV. The sequence would also be used to investigate phylogenetic relationships among the Bromoviridae, especially between PDV, CiLRV and AMV.
2.0 MATERIALS AND METHODS

2.1.0 Purification procedures

2.1.1 Virus origin

A PDV isolate was originally detected in P. avium cv. Salmo in 1971 at the Summerland Research Centre. Its identity has been confirmed by ELISA in the laboratory of Dr. G. Mink (WSU-IAREC Prosser, WA) and by indexing on herbaceous hosts (Cucurbita spp. and C. quinoa) and on the woody indicator P. serrulata cv. Shirofugen. The virus was transferred to P. mahaleb where it is maintained. This tree has been shown to be free of PNRSV by ELISA (Eastwell, unpublished results). PDV was transmitted to pumpkin, C. maxima cv. Buttercup, in the spring of 1991 by grinding young apical leaves in ice-cold 0.05 M phosphate buffer, pH 7 with 1% nicotine and rubbing sap onto pumpkin cotyledons dusted with carborundum powder. Male flowers of this pumpkin plant were used as inoculum for further pumpkin plants. Pollen was also collected, allowed to air dry for about 24 hours and stored at -70°C. This frozen pollen was used to initiate new infections of pumpkin plants as required.

2.1.2 Virus purification

Approximately 60 pumpkin seedlings were inoculated with PDV from the flowers of an infected pumpkin. After 10-14 days virus was isolated from these plants by a modified
procedure of Fulton (1959). Leaves were homogenized in 1.2 ml/g tissue ice-cold 30 mM sodium phosphate; 10 mM EDTA, pH 8.0 (PDV buffer) containing 0.14% β-mercaptoethanol (βME) and 7% (w/v) alumina powder, in a chilled Waring blender. This homogenate was clarified by centrifugation for 10 minutes at 6000 rpm in a Beckman JA 14 rotor at 4°C. The supernatant was returned to the blender and homogenized briefly with chilled hydrated calcium phosphate (approximately 5 g). This was centrifuged at 8000 rpm for 10 minutes in a Beckman JA 14 rotor at 4°C. The supernatant was filtered through miracloth and centrifuged at 42000 rpm for 2.5 hours in a Beckman 45Ti rotor at 4°C. Pellets were resuspended in 200 µl chilled PDV buffer, pooled and layered onto a 25% (w/v) sucrose cushion prepared in PDV buffer. This was centrifuged for 2 hours at 50000 rpm in a Beckman 70Ti rotor at 4°C. The pellets were resuspended and pooled as before and layered onto a 10% to 40% (w/v) linear sucrose gradient in PDV buffer and centrifuged for 2.5 hours at 38000 rpm in a Beckman SW40Ti rotor at 4°C. The gradient was scanned at 340 nm with an ISCO UA-5 scanner and a large, centrally located band was collected. These fractions were pooled and virus collected by centrifugation for 1.5 hours at 50000 rpm in a Beckman 70.1Ti rotor at 4°C. The final pellet was resuspended in a minimal volume of PDV buffer (typically 100 µl) and stored at 4°C. The amount of virus contained in these fractions was determined by spectrophotometry assuming
an extinction coefficient of 5.0 for 1 cm of a 0.1% solution at 260 nm (Halk & Fulton 1978).

### 2.1.3 Coat protein electrophoresis

The protein content of the final virus fraction was determined by a Bradford dye-binding assay (BioRad: Bradford 1976) with bovine serum albumin (BSA fraction V: Sigma Chemical Co) used as standard. The protein content of the virus samples was adjusted to approximately 5 mg/ml and it was mixed with 4 volumes of loading buffer (62.5 mM Tris-HCl pH 6.8; 10% (v/v) glycerol; 2% (w/v) sodium dodecyl sulphate (SDS); 5% (v/v) ßME; 0.001% (w/v) bromophenol blue). The sample was heated to 95°C for 2 minutes prior to loading.

For electrophoresis the method described by Laemmli (1970) using a discontinuous buffer system was used with a BioRad Mini-protean II unit. Samples were electrophoresed on a 12% polyacrylamide gel overlaid with a 4% stacking gel, at 200 V for 40 minutes in 0.1% SDS; 25 mM Tris; 200 mM glycine; pH 8.3. The following proteins were used as molecular weight markers (Dalton VII set: Sigma Chemical Co.): BSA (66.0 kDa); ovalbumin (45.0 kDa); glucose-3-phosphate dehydrogenase (36.0 kDa); trypsinogen (24.0 kDa); trypsin inhibitor (20.1 kDa); α-lactalbumin (14.2 kDa). The protein bands were visualized by staining the gel with Coomassie brilliant blue R-250 or silver nitrate (Merril 1990). Gels were dried under vacuum and photographed.
2.1.4 Genomic RNA isolation

Genomic PDV RNA was isolated from partially purified virus. Pelleted material obtained after the first ultracentrifugation step (section 2.1.2) was resuspended in 100 μl PDV buffer and extracted twice with an equal volume of water saturated phenol heated to 80°C. Two extractions using phenol:chloroform:isoamyl alcohol (25:24:1) and a final extraction with chloroform:isoamyl alcohol (24:1) followed. RNA was precipitated from the final aqueous fraction by adding 0.1 volume 3.1 M sodium acetate, pH 5.2, and 3 volumes of ice-cold 95% ethanol. The tube was left at -70°C for at least 1 hour, then centrifuged at 13000 rpm for 45 minutes at 4°C in a microcentrifuge. The pellet was washed with 70% ethanol, dried under vacuum and dissolved in 50 μl diethyl pyrocarbonate-treated water. The sample was immediately divided into 5 μl portions which were stored at -70°C. One aliquot was used to measure the RNA content by spectrophotometry and to examine its quality by electrophoresis in a 1% agarose gel containing 5 mM methylmercuric hydroxide (MeHgOH) and borate buffer (40 mM sodium borate; 1 mM EDTA; pH 8.2), as described by Sambrook et al. (1989). RNA samples were denatured in 15 mM MeHgOH for 5 minutes at room temperature prior to loading. After electrophoresis the MeHgOH was inactivated with 0.1 M ammonium acetate and the RNA was visualized by staining with ethidium bromide. Single stranded RNA molecular size standards (Sigma) were co-
electrophoresed to estimate the sizes of the genomic RNA bands and the gel was photographed to allow measurement of band migration.

2.1.5 Extraction of double-stranded (replicative form) RNA from infected leaves

Young leaves were collected from the *P. mahaleb* stock tree as soon as they became available in the spring and stored in batches of 7 g at -70°C where their dsRNA was stable for at least a year. To extract dsRNA, 7 g of leaves were ground to a powder in liquid nitrogen with a pestle and mortar and allowed to thaw in a 50 ml tube containing 9 ml water saturated phenol, 9 ml GPS buffer (0.2 M glycine; 0.1 M sodium phosphate buffer, pH 9.5; 0.6 M NaCl) and 0.5 ml βME. On thawing, 0.5 ml 20% (w/v) SDS was added and the tube placed on a rotary shaker for about 45 minutes. The samples were then centrifuged for 5 minutes in a bench-top centrifuge at 3000 rpm. The aqueous phase was transferred to a fresh tube on ice and the organic phase was re-extracted with 5 ml GPS buffer. The two aqueous phases were pooled and one third volume of 10 M LiCl, chilled to -20°C, was added dropwise whilst vortexing to ensure rapid mixing. A precipitate was allowed to form overnight at 4°C.

The sample was centrifuged in a bench-top centrifuge for 10 minutes at full speed (3000 rpm). The supernatant was aspirated into a fresh tube and 95% ethanol was added to a
final concentration of 18% along with 1.5 g cellulose powder (CC41: Whatman). The dsRNA was allowed to bind to the cellulose by shaking the tubes on ice for 30 minutes on a rotary shaker. The cellulose was washed 3 times with STE (100 mM NaCl; 10 mM Tris-HCl; 1 mM EDTA, pH 8.0) containing 18% ethanol and samples were loaded into 20 x 1 cm chromatography columns and each column washed with 300 ml STE containing 18% ethanol at a flow rate of approximately 2 ml/min. After the columns had drained completely, they were purged dry by forcing air from a syringe through the column. DsRNA was eluted from the cellulose with 3 ml 0.5 mM EDTA, pH 8.0, followed by forced air to dry the column. Two more 2 ml portions were passed through the column and the eluants pooled in a 30 ml Corex tube containing 700 μl 3 M sodium acetate pH 5.2. Three volumes of ice cold 95% ethanol were added to the tubes and they were placed at -70°C overnight.

Samples were centrifuged at 11000 rpm in a Beckman JS 13.1 rotor at -5°C for 45 minutes to pellet the dsRNA. The supernate was discarded and the pellet dried under vacuum and redissolved in 200 μl DNase buffer (0.1 M sodium acetate; 5 mM MgCl₂, pH 5.0), transferred to an eppendorf tube and incubated with 10 U deoxyribonuclease I (DNase I: Gibco/BRL) at 37°C for 20 minutes. The samples were centrifuged briefly to remove fine cellulose particles carried over from the chromatography before adding 20 μl 3 M sodium acetate, pH 5.2 and 3 volumes ice-cold 95% ethanol. DsRNA was precipitated
for several hours at -70°C or for 20 minutes in an isopropanol:dry ice bath. The precipitate was collected by centrifugation at 13000 rpm in a microcentrifuge for 45 minutes at 4°C. The supernate was removed and the pellet washed with 100 µl 70% ethanol, dried under vacuum and redissolved in 20 µl TE (10 mM Tris-HCl; 1 mM EDTA, pH 8.0). A 4 µl aliquot was analyzed by agarose gel electrophoresis. DsRNA isolated from Nicotiana glauca infected with CMV was used as a molecular weight marker.

To remove single stranded RNA (tRNA and rRNA), samples were either digested with 1 unit RNase T₁ (Pharmacia) in STE for 10 minutes at room temperature prior to electrophoresis, or the gel was placed in a solution of 2X SSC containing 50 µg/ml RNase A (Pharmacia) for 1 hour at room temperature with shaking after electrophoresis.

2.2.0 Antibody production

2.2.1 Production of polyclonal antibodies in chickens

Fifteen week old laying hens (Red Sussex) were purchased from Rump & Sendall, Vernon, BC. When the chicken to be immunized was laying reliably, ie. laying at least 5 eggs/week for 2 weeks, pre-immune eggs were collected and the chicken was given an intramuscular injection of approximately 1 mg purified PDV in Freund's complete adjuvant. After ten days, eggs were collected in groups of six. Antibody (IgY) was isolated from them using the following procedure (van
Regenmortel 1982). Yolks were separated from whites and washed in a beaker with distilled water. Their volume was measured and 3 volumes of saline buffer (5 mM sodium phosphate, pH 7.2, 0.1 M NaCl) were mixed with the yolks. The yolks were broken and the solution was brought to 3.5% (w/v) ground polyethylene glycol (PEG 6000: Sigma) and stirred on ice for 30 minutes. The sample was centrifuged in a Beckman JA 14 rotor at 10000 rpm for 10 minutes at 4°C. The supernatant was filtered through moistened cotton wool, brought to 12% (w/v) with PEG 6000 and stirred at 4°C for 30 minutes. The centrifugation was repeated and the supernatant discarded. The pellet was drained well and dissolved in saline buffer overnight.

The solution was clarified by centrifugation as above and antibodies reprecipitated with 12% PEG as before. The final pellet was dissolved in a minimal volume of saline buffer containing 0.2% sodium azide and stored at 4°C. This sample was analyzed by SDS polyacrylamide gel electrophoresis (PAGE) (section 2.1.3) and purified further by hydroxylapatite (HAP: Boehringer Mannheim) chromatography (cf. section 2.2.5) for use in ELISA. A 10 ml HAP column was equilibrated with 10 mM phosphate buffer, pH 6.8. The IgY sample was diluted ten-fold with running buffer, loaded onto the column, and eluted with a 100 to 500 mM phosphate gradient at pH 6.8 at a rate of approximately 2 ml/min. The eluant was monitored at 280 nm and 0.5 ml fractions were
Peaks were pooled and IgY precipitated by adding sodium sulphate to 14% (w/v) from a saturated solution (36% (w/v)). After 1 hour at room temperature the IgY were pelleted by centrifugation at 10000 rpm, 10 minutes in a Beckman JS 13.1 rotor. The highly soluble pellet was dissolved a minimal volume of saline buffer containing 0.02% (w/v) sodium azide and stored at 4°C.

2.2.2.0 Production of monoclonal antibodies

2.2.2.1 Immunization after cyclophosphamide treatment

Healthy pumpkin extract was prepared by harvesting 130 g healthy pumpkin plants and proceeding with the PDV isolation as detailed in section 2.1.2 until after the first ultracentrifugation step. At this stage, the pellets were redissolved in phosphate buffered saline (PBS: Harlow & Lane 1988), clarified by low speed centrifugation and stored at 4°C. The protein concentration of the supernate was determined by the Bradford assay.

Cyclophosphamide (Sigma) was dissolved in PBS (1 g/30 ml) and diluted to 16.5 mg/ml shortly before use. The healthy pumpkin extract was diluted with PBS to 2.5 mg protein/ml and injected intraperitoneally into two female Balb/c mice without adjuvant. This was followed by an intraperitoneal injection of 100 mg cyclophosphamide/kg mouse after 10 minutes, 24 hours and 48 hours (Matthew & Sandrock 1987). After the drug had been allowed to clear for 2 weeks,
the mice were given an intraperitoneal injection of PDV (250 μg) in Freund's complete adjuvant. This injection scheme was repeated after a further 2 weeks, but using Freund's incomplete adjuvant. A final intraperitoneal boost of 250 μg PDV was given without adjuvant 3 days before the fusion.

2.2.2.2 Determination of the immune response by TAS-ELISA

Tail bleeds were carried out after the final boost to determine the immune response of the mice. Approximately 100 μl of blood was collected into heparinized capillary tubes (Oxford Labware). Blood cells were pelleted by centrifugation for 5 minutes at 800 g and the clear supernatant serum was carefully removed and stored at 4°C. The serum was tested by TAS-ELISA using four wells for each mouse. Microtitre plate wells (EIA poly-styrene: Nunc) were coated with rabbit serum prepared against PDV strain 876 (PVAS 290: American Type Culture Collection) diluted 1:2000 in PBS and incubated at room temperature for 2 hours. Wells were washed three times with PBS after this and all subsequent steps. Blocking was achieved with 3% BSA, 0.05% Tween 20 in PBS for 1 hour at room temperature. Purified PDV at a concentration of 300 ng/ml in PBS with 0.5% BSA was applied to the wells to act as antigen and pumpkin cotyledons ground in PBS served as negative controls. Plates were incubated at 4°C overnight with the antigen. Serum from the
test-mice was diluted 1:250 to 1:3000 in 0.5% BSA in PBS, applied to the wells and incubated for 2 hours at room temperature. Alkaline phosphatase labelled goat-anti-mouse antiserum (Gibco/BRL) was added at the recommended concentration (1:3000) in 0.5% BSA in PBS and incubated for 2 hours at room temperature. Detection was achieved by the addition of 0.1% (w/v) para-nitrophenol phosphate in 10% (w/v) diethanolamine, pH 9.5. The microtitre plate was read in a microtiter plate reader (Titertek MCC/340; ICN) at 405 and 620 nm after 2 and 24 hour incubations under subdued light. A\textsubscript{620} readings were subtracted from A\textsubscript{405} readings.

2.2.2.3 Fusion mediated by polyethylene glycol

All work described in this section was done in a biological containment hood using sterile technique. Twenty 96-well thymocyte feeder plates were prepared the day before the fusion. Ten 6 week old Balb/c mice were sacrificed to provide thymocytes. Thymuses were removed aseptically from the mice and pooled together in a petri-dish containing 20 ml prewarmed DMEM (Dulbecco’s modified Eagle medium; Sigma). Cells were released by overlaying the organs with several layers of sterile gauze and crushing them gently with a sterile syringe plunger. Thymocytes were transferred to a centrifuge tube with a pipette and pelleted by centrifugation at 800 g for 2 minutes. The pellet was washed once with DMEM and resuspended in 100 ml DMEM containing 20% foetal calf
serum (FCS: Gibco/BRL) and 50 μg/ml gentamycin (Sigma).
Thymocytes were plated into twenty 96-well plates, 50 μl per well, and placed into a 37°C incubator with a 10% CO₂ atmosphere.

NS1 myeloma cells were also prepared at least 2 days before the fusion. These had been stored in liquid nitrogen and were thawed by immersing the storage-cryovial in tepid water until the cells had just thawed. Cells were immediately diluted in 10 ml DMEM, pelleted at 800 g, washed again and resuspended in 30 ml DMEM containing 10% FCS and 50 μg/ml gentamycin. Cells were seeded into a 15 cm petri dish and placed in the CO₂ incubator overnight. The next day, the NS1 cells were resuspended and half (15 ml) transferred to a new petri dish. A further 15 ml DMEM was added to each dish and the cells were allowed to grow to 80% confluency.

The fusion was performed essentially as described by Harlow & Lane (1988). The mouse with the higher specific immune response, based on the tail-bleed TAS-ELISA, was sacrificed and its spleen removed under aseptic conditions. Splenocytes were extracted using the same procedure described for thymocytes. The cells were pelleted at 800 g in a clinical centrifuge and washed once with DMEM. Special care was taken to remove any blood clots and connective tissue present. The NS1 cells from two petri dishes estimated to be 80% confluent were harvested and washed twice with DMEM
taking care to remove as much media as possible. The two cell types, the splenocytes and the NS1 myelomas, were then mixed in DMEM and pelleted together. Care was taken to remove as much of the super-natant as possible. The pellet was resuspended by adding 0.9 ml of a solution of PEG 3000 (Sigma) previously mixed 1:1 with DMEM and warmed to 37°C, over a period of 1 minute with gentle stirring. The cells were stirred for a further minute, followed by the addition of 1 ml DMEM over 1 minute and 9 ml DMEM over 2 minutes with continuous gentle stirring. After this, the cells were immediately centrifuged for 5 minutes at 400 g and resuspended in 10 ml DMEM containing 20% FCS and gentamycin. This was mixed with a further 100 ml of the same medium and plated out onto the feeder plates at 50 μl per well. After approximately 10 hours at 37°C in the CO₂ incubator, 25 μl of 5X HAT selection medium was added to each well, (1X HAT is 100 μM hypoxanthine, 16 μM thymidine, 0.4 μM aminopterin in DMEM with 20% FCS and 50 μg/ml gentamycin). The HAT medium was replaced by HT medium (containing 100 μM hypoxanthine and 16 μM thymidine) after approximately 2 weeks.

The hybridomas were left undisturbed in the CO₂ incubator for 1 week. After this period, wells were inspected for hybridomas under an inverted microscope and all wells containing one or more hybridomas were marked. Wells were screened for anti-PDV antibodies by TAS-ELISA at this stage. The assay was the same as described for tail bleeds.
(section 2.2.2.2) except that 50 µl tissue culture supernatant (TCS) was removed aseptically from each well to be assayed for use in the mouse-anti-PDV antibody step in the ELISA. Each well was assayed using PDV-infected and healthy cucumber cotyledons as antigen. After removal of the TCS from the tissue culture plates, it was immediately replaced with fresh DMEM containing 20% FCS, gentamycin and HAT. Hybridomas in wells giving positive values by TAS-ELISA against PDV-infected material were transferred to 24-well plates and were single cell cloned by limiting dilution (Harlow & Lane 1988).

2.2.3 Single cell cloning

Ninety-six well microtitre plates with thymocyte or spleenocytes feeder cells were prepared for single cell cloning as described in section 2.2.2.3. Each plate could accommodate two cell lines for single cell cloning. Hybridomas to be single cell cloned were pipetted into well A1 (or E1) of a plate. Serial dilutions (1:1) were made first down the column and then across the rows of the plate. The plates were returned to the incubator and left until hybridoma colonies became visible. The numbers of hybridomas per well was marked on the lids of the plates. At this point plates were screened by TAS-ELISA as described above and one positive well per plate containing a single hybridoma was subjected to another round of single cell cloning. If there
were no wells with single, positive hybridomas on a plate, the positive well with the fewest hybridomas was single cell cloned, until two rounds had been completed successfully. Clonal hybridomas were grown to high density in 24-well, then 12-well plates and finally in 50 ml tissue culture flasks. At this stage cells were also frozen in liquid nitrogen for safe, long-term storage (section 2.2.4). A monoclonal antibody called PDA-3C was identified at this stage.

2.2.4 Cryogenic storage of hybridomas

Hybridomas to be frozen were grown until 80% confluent in 15-20 ml DMEM supplemented with 20% FCS and 50 μg/ml gentamycin. Cells were harvested by flushing them out of the T-flask with a 25 ml pipette being sure to wash them off the bottom of the flask. They were transferred to a centrifuge tube, pelleted at 800 g for 5 minutes, resuspended in 1 ml of prechilled 46% DMEM, 46% FCS, 8% dimethyl sulphoxide and transferred to prechilled cryovials (Nunc). These were placed into an insulated box at -70°C to allow them to cool slowly (approximately -1°C/min) overnight. After this they were sealed with cryoflex tubing (Nunc) and transferred to liquid nitrogen for storage.

2.2.5 Isolation of antibody from tissue culture supernatant and isotyping

Hybridomas were grown in 15 ml culture flasks until 80%
confluent. At this stage they were resuspended with a 25 ml pipette and approximately 80% of the volume transferred to a centrifuge tube. The remaining hybridomas were returned to the flask and the media replaced. Cells were removed from the collected media by centrifugation and the supernatant stored at 4°C until required. The flask was harvested until about 150 ml of TCS had been collected. The monoclonal antibody (PDA-3C) was isolated from the TCS by HAP chromatography (Harlow & Lane 1988). A 10 ml HAP column was equilibrated with 10 mM sodium phosphate, pH 6.8 at a flow rate of 2.5 ml/min. The TCS was loaded in 3 batches of 50 ml onto a single column and eluted until a peak, monitored at 280 nm, had been removed. Bound antibody was removed from the HAP by elution with 200 mM sodium phosphate, pH 6.8. A single peak was collected. All antibody containing fractions were pooled, chilled to 4°C and the antibody precipitated by dropwise addition of an equal volume of chilled, saturated ammonium sulphate, pH 7. The sample was precipitated at 4°C for 1 hour and centrifuged in a Beckman JA 14 rotor at 10000 rpm for 10 minutes, 4°C. The pellet was redissolved in a minimal volume (approximately 1 ml) 0.02% sodium azide in PBS and stored at 4°C. This fraction was used for all serological assays requiring a monoclonal antibody. The column was regenerated by washing with 1 M NaCl and re-equilibrating with 10 mM sodium phosphate, pH 6.8.

The isotype of PDA-3C was determined using an
erythrocyte agglutination assay kit (Serotec). One column (8 wells) of a 96-well plate with U-bottom wells was filled with 30 μl of the antiserum to be tested diluted 1:50 with PBS. This was mixed with an equal volume of each specific isotyping reagent to be tested (anti-IgG<sub>1</sub>; -IgG<sub>2a</sub>; -IgG<sub>2b</sub>; -IgG<sub>3</sub>; -IgA; -IgM, all linked to sheep erythrocytes,) as well as positive and negative control wells. The plates were tapped gently to mix and left covered on a flat, stable surface for 1 hour. A small red button at the bottom of the well was considered negative; a partial or full carpet of agglutination was considered positive. If all wells failed to agglutinate, the assay was repeated using higher concentrations of the test-solution.

2.2.6 Western blot

A sample of PDV coat protein was electrophoresed as described in section 2.1.3. After electrophoresis, the gel was soaked in transfer buffer (50 mM Tris-HCl, pH 7.6; 380 mM glycine; 0.1% SDS; 20% methanol). Nitrocellulose (NC: Schleicher & Schuell) was cut to the same size as the gel, wetted in distilled water and soaked briefly in transfer buffer. The NC was overlaid onto the gel and sandwiched between three layers of Whatman 3MM paper, taking care to remove all air bubbles in the sandwich. This was placed into an electroblot transfer apparatus and the proteins were electrophoretically transferred overnight to the NC at 4°C.
with a constant voltage set to 60 V. After the transfer the gel was removed and stained with Coomassie brilliant blue R-250 to verify transfer and the NC was immersed in isopropanol for 1 minute before being allowed to dry completely.

The NC was then rewetted in distilled water, blocked in 3% skim milk powder (Carnation) in PBS with 0.05% Tween-20 for 20 minutes and transferred to a plastic containing 2 ml PDA-3C diluted 1:1000 with 0.5% skim milk powder in PBS and left at 37°C for 1 hour. The NC was then removed from the bag, washed in three changes of PBS and incubated with alkaline phosphatase-linked goat-anti-mouse antibody (Gibco/BRL) diluted 1:1000 (section 2.2.2.2). After three further washes in PBS, the NC was washed in PBS, pH 9.5 and transferred to a solution of 0.033% nitro-blue tetrazolium, 0.017% 5-bromo-4-chloro-3-indolyl phosphate in TBS, pH 9.5 with 5 mM MgCl₂. Colour development was allowed to proceed for at least 20 minutes in subdued light. The reaction was terminated by transferring the blot to a solution of 10 mM EDTA, pH 8.0. Results were photographed.

2.2.7 Conjugation of PDA-3C to alkaline phosphatase

A maleimide alkaline phosphatase kit (Pierce) was used to conjugate monoclonal PDA-3C to alkaline phosphatase (AP). PDA-3C, 6.25 mg, was precipitated with an equal volume of (NH₄)₂SO₄, redissolved in 1 ml TBS and desalted over a beaded
polyacrylamide column (Pierce kit). One-half ml fractions were collected and their protein contents determined by the Bradford dye-binding assay. The two fractions with the highest protein contents were pooled to yield 2 mg protein/ml. Six mg 2-mercapto ethylamine (MEA) was added and allowed to react at 37°C for 1 hour. Activated AP was prepared by adding 30 µl 2.9% (w/v) [sulpho succinimidyl 4- (N-maleimidomethyl) cyclohexane-1-carboxylate] to 5 mg AP. This was allowed to react for 30 minutes at room temperature and then desalted over a cross-linked dextran column (Pierce kit) equilibrated with TBS supplemented with 0.15 M NaCl, 2 mM ZnCl₂, 4 mM MgCl₂, pH 7.6. One-half ml fractions were collected and their protein contents determined as before. Mixing 0.5 mg MEA-reduced monoclonal with 2 mg activated AP in supplemented TBS buffer, initiated conjugation which proceeded at room temperature for 1 hour. The resulting conjugate, PDA-3C-AP was stored at 4°C.

PDA-3C-AP was assayed by DAS-ELISA. Microtiter plates (Linbro EIA) were coated for 2 hours at room temperature with PDA-3C in PBS at dilutions of 1:100 to 1:3000. Plates were blocked and antigen applied as described in section 2.2.2.2. PDA-3C-AP was applied in 0.5% BSA, PBS at dilutions of 1:100 to 1:3000 and left for hours at room temperature. After three washes with TBS, substrate was applied and the plates read at 405 and 620 nm after 2 hrs and 24 hrs.
2.2.8 Production of F(\(\text{ab}'\))\(_2\) fragments from PDA-3C

Approximately 1 mg hydroxylapatite-purified PDA-3C in 225 \(\mu\)l, was incubated with 2.5 \(\mu\)l (0.5 \(\mu\)g) pepsin (porcine mucosa: Sigma) in 100 mM sodium citrate buffer, pH 3.5, overnight at 37°C (Harlow & Lane 1988). The reaction was terminated by the addition of 0.1 volume 3 M Tris-HCl, pH 8.8. The sample was then centrifuged to pellet denatured protein and the supernatant analyzed by SDS PAGE. Native samples, and samples reduced with \(\beta\)ME before loading, were loaded side-by-side on the gel. Electrophoresis was performed as described in section 2.1.3 and the gels were stained with silver nitrate. The F(\(\text{ab}'\))\(_2\) fragments were purified further by diethylaminoethyl (DE-52: Whatman) ion exchange chromatography to remove F\(_c\) fragments and intact antibodies. A 15 ml column was prepared and equilibrated with 10 mM Tris-HCl, pH 8.5. The digested antibody solution was neutralized with 100 mM Tris-HCl, pH 8.5, diluted 10-fold with distilled water and loaded onto the column at 1 ml/min. The column was washed with 10 mM Tris-HCl, pH 8.5 and the eluant monitored at 280 nm. When the baseline had returned to zero, a 50 to 500 mM NaCl gradient in column buffer was applied and all peaks collected. F(\(\text{ab}'\))\(_2\) fragments were concentrated by ammonium sulphate precipitation. The column was regenerated by washing sequentially at a flow rate of 1 ml/min with 30 ml each of 1 M NaCl; 0.5 N HCl; 0.5 N NaOH; then 100 ml 150 mM Tris-HCl, pH 8.5 and finally 100 ml 10 mM
Tris-HCl, pH 8.5.

The purified F(ab')₂ were assayed for their ability to trap PDV by TAS-ELISA by loading them onto a microtitre plate (1:10-1:1000) in PBS and allowing them to bind for 2 hours at room temperature. Plates were blocked and antigen applied as described in section 2.2.2.2. PDA-3C, AP-labelled sheep-anti-mouse (anti-Fc region: Kirkegaard Perry Labs) antibody, diluted as recommended by the manufacturer, and substrate were added as set out in section 2.2.2.2 and the absorbances read at 405 and 620 nm after 2 and 24 hours.

2.2.9 Detection of PDV by TAS-ELISA, RT-PCR and bioassay in sweet cherry

To evaluate the monoclonal PDA-3C for routine field indexing by TAS-ELISA (triple-antibody sandwich ELISA), 40 sweet cherry trees showing symptoms suggesting a viral infection (eg. shot-holes, leaf discolouration) were selected for analysis. They were indexed by RT-PCR, TAS-ELISA and the 'Shirofugen' bioassay. The RT-PCR was performed as described in section 2.3.15; the bioassay was done by T-budding two buds of each sample-tree onto a 'Shirofugen' indicator tree in the spring. These were inspected after 4 and 6 weeks for the presence of gumming at the bud union which would indicate the presence of ilarvirus. For the TAS-ELISA, six young leaves were taken at random from each tree and assayed in
duplicate on separate plates using the assay described in section 2.2.2.2. In addition, 15 symptomless trees were selected to act as negative controls for the TAS-ELISA. These trees were also tested by RT-PCR and by the bioassay. Since the bioassay cannot distinguish between PDV and PNRSV, all trees which tested positive on the bioassay were also assayed for PNRSV by TAS-ELISA using PVAS-22 (American Type Culture Collection) rabbit serum at a dilution 1:500 to trap virus and monoclonal antibody UCB 1332 (a gift from D. Opgenorth) diluted 1:25, and AP-linked goat anti-mouse serum (Gibco/BRL) to detect PNRSV. Buffers and incubation times were the same as described for the PDV TAS-ELISA.

The results of the RT-PCR and the bioassay on 'Shirofugen' established that the 15 negative control trees were free from PDV. To establish the threshold value for TAS-ELISA, leaves from these healthy trees were included in each TAS-ELISA for the test trees. The sum of the mean plus four standard deviations of the healthy control population ($x_n + 4S$) was used as the threshold value for a positive reaction in the test population (Sutula et al. 1986). To normalize for slight differences between plates, all data were expressed as the ratio of the test value to the threshold value associated with that plate. The ratios for duplicate samples were averaged and the values used for further analysis. A Chi-squared dispersion test (Maxwell 1961) was used to test whether the proportion of leaves that
would show a positive reaction by TAS-ELISA was the same for all positive trees.

2.3.0 cDNA library of PDV

2.3.1 Preparation of cDNA: first strand synthesis

Single-stranded genomic RNA was isolated from partially purified PDV particles as described in section 2.1.4. A 3 µg sample was heated to 65°C in the presence of 450 ng random hexamers (pd(N)_6) and cooled on ice. A first strand reaction mix was prepared separately and added to the RNA to give the following conditions: 50 mM Tris-HCl, pH 8.3; 40 mM KCl; 6 mM MgCl_2; 1 mM dithiothreitol (DTT); 0.1 mg/ml BSA and 0.75 mM each dNTP in a final volume of 25 µl. This was equilibrated to room temperature and 50 U Superscript II™ reverse transcriptase (Gibco/BRL) was added, mixed gently and allowed to react for 10 minutes at 25°C to allow primer extension. The reaction was then warmed to 48°C, a further 200 U RTase were added and it was incubated for 1 hour. A 1 µl sample of this reaction was removed immediately and added to 0.5 µl [α_32P]dCTP (5 µCi) and run in parallel with the main reaction. This pilot first strand reaction was terminated by the addition of 0.5 µl 0.5 M EDTA, pH 8.0. The first strand products were separated by electrophoresis on an alkaline agarose gel (Sambrook et al. 1989) and autoradiographed by wrapping the gel in Saran wrap and placing it onto a sheet of Kodak X-omat AR film overnight.
2.3.2 Second-strand synthesis

After 1 hour, the main reaction was chilled on ice and combined with 136 µl of second strand reaction mix to give the following final conditions: 40 mM Tris-HCl, pH 7.5; 10 mM MgCl₂; 10 mM (NH₄)₂SO₄; 100 mM KCl; 50 µg/ml BSA; 0.5 mM dNTPs; 5 mM DTT; 0.15 mM β-NAD⁺; 40 U E. coli DNA polymerase I (Gibco/BRL); 10 U E. coli DNA ligase (Gibco/BRL) and 5 U E. coli RNase H (Gibco/BRL). A 10 µl sample was immediately removed from the reaction and mixed with 1 µl (10 µCi) [α³²P]dCTP. Both reactions were incubated at 12°C for 1 hour followed by 4 hours at 20°C.

The second strand reactions were terminated by the addition of 0.5 M EDTA, pH 8.0, to 20 mM. The products of the main reaction were precipitated in ethanol and ammonium acetate, the pilot reaction products were analyzed by agarose gel electrophoresis and autoradiography.

2.3.3 End polishing reaction

After precipitation, the cDNA pellet was washed with 70% ethanol, dried and redissolved in 7.8 µl water and 1 µl 10X Pfu buffer (1X Pfu buffer is 10 mM KCl; 6 mM (NH₄)₂SO₄; 20 mM Tris-HCl, pH 8.0; 2mM MgCl₂; 0.1% Triton X-100 and 10 µg/ml BSA: Stratagene), 2 µl 10 mM dNTP mix and 0.2 µl Pfu polymerase (0.5 U: Stratagene). This was overlaid with mineral oil and incubated at 72°C for 20 minutes. The reaction was terminated by the addition of 0.5 M EDTA,
pH 8.0, to 20 mM and stored at -20°C until required. The DNA content of this sample was estimated by measuring the emission of a 1 μl sample in a fluorometer, using Hoechst Dye 33342 (Hoeffer Scientific Instruments). To select large cDNA molecules, the entire sample was loaded onto a single well in a 1% agarose gel and electrophoresed for 15 minutes at 60 V. Suitable molecular weight markers (eg. 1 kb ladder: Gibco/BRL) were also run. The gel was stained with ethidium bromide and the region of the gel containing the required size of cDNA was excised from the gel on a long wavelength UV transilluminator. The cDNA was extracted from the gel slice using the Qiaex gel extraction kit (Qiagen).

2.3.4 Blunt-end ligation

The results of the autoradiographs of the first and second strand products were used to estimate the average lengths of the cDNA products and the fluorometer reading was used to calculate the concentration of cDNA. Using this information, a ligation reaction containing pBluescript SK⁺ (Stratagene) or pGem4Z (Promega) cut with EcoRV or SmaI respectively and dephosphorylated with calf intestinal phosphatase (CIP: Gibco/BRL) was prepared using a 5:1 molar ratio of vector:cDNA. The reaction conditions were: 50 mM Tris-HCl pH 7.6; 10 mM MgCl₂; 1 mM ATP; 1 mM DTT; 5% (w/v) PEG 8000 and 10 U T4 DNA ligase (Gibco/BRL). The reaction was allowed to proceed overnight at 16°C. The plasmids were
then transformed into competent *E. coli* DH5α by one of the two methods described below (section 2.3.5) and plated onto 2X TY (1.6% bacto-tryptone, 1% yeast extract, 0.5% NaCl, 2% agar) plates containing 200 μg/ml ampicillin and spread with 800 μg X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) and 800 μg IPTG (isopropylthio-β-galactoside).

### 2.3.5 Preparation of competent *E. coli*

Competent cells for transformation by electroporation or by heat shock were prepared in bulk and stored in small aliquots at -70°C. Cells were prepared as follows. *E. coli* DH5α were inoculated to 2X TY plates overnight. A single colony was transferred into 5 ml 2X TY media and incubated overnight at 37°C in a rotary shaker. This was used to inoculate 200 ml 2X TY medium in a 11 flask and the cells grown until *A*₆₀₀ of the culture measured 0.6. The flasks were immediately immersed into an ice-water bath and chilled for 15 minutes. The cells were harvested by centrifugation at 3500 rpm in a JA14 rotor (Beckman) for 10 minutes at 4°C. The supernatant was discarded, draining away as much of it as possible. Electroporation-competent cells were resuspended in 100 ml prechilled distilled, sterile water; heat-shock competent cells were resuspended in 40 ml chilled 30 mM potassium acetate, 50 mM MnCl₂, 100 mM KCl, 10 mM CaCl₂, 15% glycerol (filter-sterilized). Both cell-types were recentrifuged as before and the supernatants again drained.
carefully, keeping them on ice as much as possible. Cells for electroporation were suspended gently in 2 ml distilled, sterile water and transferred into 1.5 ml microcentrifuge tubes on ice in 60 μl aliquots, to be used immediately or stored at -70°C. Cells for heat shock-transformation were resuspended in 8ml chilled 10 mM Na-morpholinepropane sulphonate, pH 7.0; 75 mM CaCl₂; 10 mM KCl and 15% glycerol (filter-sterilized) after the second centrifugation and divided into aliquots as described above. The transformation efficiency of the electroporation-competent cells was determined by calculating the number of colonies formed after electroporation of 1 pg, 10 pg and 100 pg pBluescript SK⁺ using a cuvette with a 1 mm gap at 1800 V (Genepulser™: BioRad). After electroporation the cells were incubated in 1 ml 2X TY medium supplemented with 10 mM MgCl₂, 10 mM MgSO₄ and 40 mM glucose at 37°C in a rotary shaker. Cells were then spread onto a 2X TY agar plate containing 200 μg/ml ampicillin and allowed to form colonies overnight. Colonies were counted the next day and the transformation efficiency calculated as the number of colonies formed per μg plasmid used. Heat shock-competent cells were transformed with the same plasmid by allowing a 40 μl aliquot of the cells to thaw on ice in the presence of the plasmid for 30 minutes with occasional gentle mixing. The tubes were then heated to 37°C for 1 minute, returned to ice for 2 minutes and transferred to 1 ml 2X TY medium and placed in a rotary shaker at 37°C
for 1 hour before being plated as described above. Colonies were counted the next day and the transformation efficiency determined.

2.3.6 Screening colonies for inserts by PCR

After overnight incubation on IPTG/Xgal plates, some white colonies (typically 10) were selected at random from each cDNA plate and screened for inserts using PCR. Colonies were picked with a sterile disposable pipette-tip and briefly immersed in 20 µl PCR mix (2 µl 10X Taq buffer (Stratagene), 2 µM M13 universal and reverse primers, 0.2 mM each dNTP, 0.2 U Taq DNA polymerase (Stratagene) overlaid with 20 µl mineral oil). These were heated to 94°C for 2 minutes and then given 30 cycles of 50°C, 30 sec (annealing); 72°C, 1.5 min (extension); 94°C, 30 sec (denaturation), in a thermocycler (Techen: PHC2). PCR products were visualized by electrophoresis on a 1% agarose gel run for 1 hour at 60 V, stained with ethidium bromide and photographed. Molecular size markers were either the 1 kbp ladder, 100 bp ladder or ΦX 174 RF DNA cut with Hae III (all from Gibco/BRL).

2.3.7 Identifying inserts using northern blots

To identify the viral RNA corresponding to cDNA inserts, it was necessary to hybridize the cDNA to dsRNA in northern blots. Northern blots were prepared by electrophoresis of PDV dsRNA as described in section 2.1.5. After
electrophoresis the gel was denatured by soaking in 0.4 N NaOH, 1 M NaCl (two 10 minute washes), neutralized in 1 M Tris-HCl, pH 7.0; 1 M NaCl for 20 minutes and placed in an electroblot apparatus. The dsRNA was transferred to a nylon membrane (Gene screen™: DuPont) in 25 mM sodium phosphate buffer, pH 6.5, overnight at 12 V, 4°C. The dsRNA was cross-linked to the membrane by a 5 minute exposure to UV light. The blot was prehybridized in 5 ml 50% deionized formamide; 1X Denhardt’s solution; 50 mM Tris-HCl, pH 7.5; 1.0 M NaCl; 1% (w/v) SDS and 10% dextran sulphate, at 42°C for 1 hour in a rotary hybridization oven.

To prepare probes, PCR products (section 2.3.6) were digested with restriction enzymes whose sites closely flanked the insert cDNA to be investigated and the insert was gel-purified using Qiaex beads (Qiagen). The purified cDNA fragment was diluted to approximately 50 ng/μl and labelled with [α³²P]dCTP by random primer labelling with 2 U Klenow fragment (Sambrook et al. 1990). The labelled cDNA probe was purified with a spin column (Sephadex G-10: Pharmacia), denatured by boiling and hybridized to dsRNA immobilized on the nylon in the prehybridization solution overnight at 42°C in a rotary hybridization oven.

The blot was washed twice at room temperature with 2X SSC (standard saline citrate is 150 mM NaCl; 5 mM sodium-citrate, pH 7.0), 0.1% (w/v) SDS for 5 minutes per wash, then twice more with the same but at 65°C and finally twice with
0.5X SSC, 0.1% (w/v) SDS at 65°C. The blot was kept damp, wrapped in Saran wrap and autoradiographed overnight at room temperature, or at -70°C with an intensifying screen if added sensitivity was required. Kodak X-omat AR film was used.

2.3.8 Preparation for sequencing

All clones which hybridized to PDV dsRNA were prepared for sequencing by preparing a high quality stock of the cDNA in its vector using commercially available plasmid miniprep kits (eg. Magic™ Minipreps: Promega; Qiagen minipreps: Qiagen). The smallest of these clones, with approximately 300 to 500 bp cDNA inserts, were sequenced first using the dideoxy chain termination method (Sanger et al. 1977) and the Sequenase™ version 2.0 kit (Amersham). Two μg of the plasmid to be sequenced was denatured with 2 N NaOH and sequenced from the M13 universal and reverse primers following the recommendations of the kit, but using [α³²P]dCTP instead of one of the recommended nucleotides. This necessitated substituting dATP for dCTP in the labelling mix supplied in the kit. All other instructions and recommendations of the kit were followed. Electrophoresis was carried out on the same day as the sequencing reactions, since the ³²P-labelled strands of DNA were unstable. Samples were electrophoresed on a 0.1 mm thick 8% polyacrylamide gel with 8.3 M urea in 1X TBE (0.89 M Tris; 1.12 M borate; 25 mM EDTA, pH 8.0) at 50 W for approximately 5 hours (long run)
and 2.5 hours (short run). After electrophoresis, the gel was transferred to Whatman paper, dried under vacuum and autoradiographed at room temperature overnight using Kodak X-omat AR film.

2.3.9 Exo III deletions

cDNA fragments longer than about 500 bp could not be sequenced entirely from the M13 universal and reverse primers on the plasmid. A subset of deletion clones was made as follows. Approximately 5 μg of the plasmid to be sequenced was digested with two neighbouring restriction enzymes in the polylinker. To ensure unidirectional deletion, an enzyme which creates a 3'-overhang was used adjacent to the primer site and an enzyme creating a 5'-overhang was used adjacent to the insert-cDNA to be deleted. Enzymes which did not cut the cDNA insert internally were used. On completion of the digest, the linearized plasmid was extracted with phenol, precipitated in ethanol and redissolved in 40 μl 66 mM Tris-HCl, pH 8.0; 6.6 mM MgCl₂ and allowed to equilibrate to 25°C. Meanwhile 15 tubes containing 2.5 μl S1 nuclease mix (40 mM potassium acetate, pH 4.6; 330 mM NaCl; 1.4 mM ZnCl₂; 6.7% glycerol and 0.3 U S1 nuclease) were prepared and chilled on ice. The number of tubes used varied depending on the size of the insert; 15 tubes were enough for a 1.5 kb insert, assuming a deletion rate of approximately 100 bp/min.

The Exo III reaction was started by adding 500 U Exo III
(Gibco/BRL) to the plasmid prep at 25°C. The reaction was mixed well and after an initial 20 second lag period, 2.5 μl fractions were removed at 1 minute intervals and transferred to the S1-mix tubes. When all the necessary time points had been taken, the tubes were warmed to room temperature and the S1 reaction allowed to proceed for 30 minutes. The reaction was terminated by the addition of 1 μl 0.05 M EDTA, pH 8.0 and tubes were heated to 80°C for 10 minutes to denature the S1 nuclease. The DNA ends were made flush with Klenow (2 U per tube in 20 mM Tris-HCl, pH 8.0; 100 mM MgCl₂; 0.125 mM each dNTP) for 5 minutes. This was followed by the addition of 40 μl T4 DNA ligation mix (prepared using Gibco/BRL 5X ligase buffer, see section 2.3.4) and 1 U T4 DNA ligase per reaction. Reactions were left at room temperature for 4 hours and then transformed into competent E. coli DH5α (section 2.3.5) and plated onto IPTG/X-gal plates (section 2.3.4).

White colonies were screened for insert size by PCR (section 2.3.6). Colonies with inserts sized approximately 100-200 bp apart were selected so that the smallest insert was ≤200 bp in size and the largest was about 150 bp smaller than the original cDNA insert. These were sequenced as described above using the primer adjacent to the restriction site used to initiate the Exo III deletions. Exo III deletion clones were made from both ends so that both strands of each clone could be sequenced.
2.3.10 Sequence alignment

Sequence was entered into the computer using the XESEE Version 3.0 programme (Cabot & Beckenbach 1989). This programme was used to align overlapping deletion fragments and clones and to check for mismatches among clones. Care was taken to ensure that all regions were sequenced at least twice and that all mismatches could be resolved satisfactorily. Sequences were also checked against the database maintained by the National Center for Biotechnology Information (NCBI) using the BLASTx and BLASTn programmes.

2.3.11 cDNA cloning of PDV RNA1

A short cDNA fragment 353 bp in length, hybridized to PDV RNA1 on a Northern blot. The sequence of this fragment was used to construct the following cDNA synthesis primer specific for RNA1: CGTAATCAACCAAT (position 1244 on RNA1; all primers were synthesized by the Core DNA Facility, University of Calgary). This was used to prime the first strand synthesis of cDNA from total viral RNA in a reaction similar to the one described in section 2.3.1 but substituting the specific primer for random hexamers. This yielded several large clones of cDNA (ca. 1.3 kb in size). Two of these were sequenced completely as described above and the information used to construct a primer for RACE PCR (rapid amplification of cDNA ends, section 2.3.12). The larger of these, 1311 bp in length was designated pPDV33.
2.3.12 The sequence of the 5’ end of PDV RNA1

A modified procedure of Hirzmann et al. (1993) was used to determine the sequence of the 5’-terminus of RNA1 up to and including the cap structure. Two primers were synthesized: CGGATCCAGTAAGCGGTGAG (position 316) and CGGGAT(C)$_{10}$. The former is complementary to the region approximately 300 bp from the 5’-end of the known sequence on pPDV33. Both primers have a BamHI site (GGATCC) at their 5’ end to facilitate cloning of RACE fragments.

The RACE PCR was preceded by a first-strand synthesis reaction using randomly primed total viral RNA exactly as described in section 2.3.1. After the RTase reaction the first strand products were extracted with phenol:chloroform and precipitated in ethanol from ammonium acetate. The resulting pellet was resuspended in 19 µl water and 5 µl 5X tailing buffer (5X tailing buffer is 0.5 M potassium cacodylate, pH 7.2; 10 mM CoCl$_2$: Stratagene) and incubated at 37°C with 0.2 mM dGTP and 5 U terminal deoxynucleotidyl transferase (TDT from calf thymus: Stratagene) for 20 minutes. The reaction was then placed on ice and 1 µl added to a PCR mix containing 2 µM of each RACE primer, 0.2 µM each dNTP in Taq buffer (Stratagene) as well as 0.1 U Taq DNA polymerase. The DNA was amplified using 35 cycles of denaturation at 94°C, annealing at 40°C and extension at 72°C for 30 seconds each. The 350 bp product was digested with BamHI and purified after gel electrophoresis (using Qiaex...
beads). The fragment was ligated overnight at room
temperature into BamHI-cut pBluescript SK' (section 2.3.4)
which had been treated with CIP and transformed into
competent E. coli DH5α (section 2.3.5). Colonies were
selected for inserts on IPTG/X-gal plates as before and white
colonies were screened for 350-bp inserts by PCR using the
M13 universal and reverse primers (section 2.3.6).

Twelve clones of the correct size were selected and
sequenced from the reverse primer (section 2.3.8). Since the
ligation was not directional, some of these had to be
sequenced again from the M13 universal primer to be able to
determine their 5'-sequence.

2.3.13 Cloning the 3' region of PDV RNA1

Another cDNA library was made from random hexamers as
described in sections 2.3.1 - 2.3.3. White colonies from this
library were transferred to a 15 cm petri dish marked with a
grid with space for 150 colonies. These were allowed to grow
overnight and transferred to nylon (Genescreen™ Plus: Du
Pont) as described by Sambrook et al. (1989). The
transferred colonies were denatured in 1% (w/v) SDS; 0.5 N
NaOH; 1.5 M NaCl, neutralized in 1 M Tris-HCl, pH 8.0; 1.5 M
NaCl. DNA was bound to the nylon by illumination with UV
light for 5 minutes. Bacterial debris was removed by washing
the nylon in several changes of 2X SSC; 0.1% SDS. The nylon
was dried and prehybridized as described by Sambrook et al.
(1989). A probe was prepared from pPDV33 (section 2.3.11) by digestion with EcoRV and PstI. This released a fragment approximately 360 bp in length from the 3'-end of the clone, which was purified after gel electrophoresis using Qiaex beads. The EcoRV fragment was labelled with \([\alpha^{32}P]dCTP\) as described in section 2.3.7, hybridized to the colony-lift and autoradio-graphed. Colonies with cDNA which hybridized to the probe were restreaked onto fresh plates and plasmid was purified from them. Two clones containing cDNA inserts 1.5 and 2.0 kb were sequenced completely.

This strategy was repeated using a PvuII/HindIII fragment from the 3'-end of the known sequence to rescreen the cDNA library above. Clones representing RNA1 sequence to position 3165 were obtained and two RACE primers were constructed based on this sequence. The 3'-terminus of RNA1 was amplified by 5'-RACE PCR (section 2.3.12) using denatured dsRNA as a template for the first strand synthesis (Coffin & Coutts 1990) primed with the specific primer CCTATAATGGGAGCTTG (position 3044). The first strand product was amplified by PCR using the nested primer CTGGAGGGGATAATGAATG (position 3118) and CGGGAGT\((C)_{10}\). The PCR product was blunt-end ligated into pBluescript (EcoRV-site) and sequenced. The sequence of the 5'-terminus of the negative strand (of the dsRNA) was taken to be the complement of the 3'-end of PDV RNA1. Ten clones were sequenced to ensure that the 3'-end had been reached. The RACE PCR was repeated using the nested
primer pairs above with \( (G)_{12} \) to amplify poly-(C) tailed first strand cDNA, to confirm the identity of the terminal nucleotide.

2.3.14 Sequence comparisons and phylogeny

The sequence obtained was compared to existing sequences on the GenBank database using the BLASTx programme at the National Center for Biotechnology Information (NCBI). The following RNA1 sequences were obtained from GenBank and used for comparison: CiLRV (accession number U23715); AMV (L00163); CMV (D12537); BMV (X02380) and RBDV (S51557).

These were aligned using the PILEUP software in the Wisconsin Genetics Computer Group package (GCG: Devereux et al. 1984). RBDV RNA1 encodes a polyprotein (Ziegler et al. 1992) and only the 5'-region of the RNA and the C terminal of the translation product were used for further analysis. Since PILEUP did not always yield optimal alignments, further editing was carried out manually with XESEE using results from pairwise alignments (BLASTx and GAP) as a guide. A phylogeny was created from the final alignment using the Phylip 3.5c software (Felsenstein 1989). Nucleic acids and their translation products were compared by using SEQBOOT to generate 100 bootstrap replicates which were analysed using DNAPARS (or PROTPARS for amino acid sequences). CONSENSE was used to generate a consensus phylogeny. This analysis was repeated using the sequence of RNA3 and of ORF3a of the
following viruses: AMV (K02703); ApMV (U15608); BMV (J02042);
CiLRV (U17390); CMV (D00385); PNRSV (L38823); PDV (L28145).

2.3.15 RT-PCR assay

Two primer pairs were chosen from the RNA3 sequence data. They were: CACGGACTTTTCATGGCGTAA and CCCTCCTGCTGGT
TTTCTTA (pair #1) as well as ACACCAAAAGCTTTCTTGTC
AACTTTGAGATCTCCCGATTG (pair #2). Pair #1 was chosen from a
region covering parts of RNA3 and RNA4 and yielded a product
179 bp in length. Pair #2 was from RNA3 only yielding a
product ca. 295 bp in length. The RT-PCR of leaf tissue was
based on the method of Wetzel et al. (1991). Tissues
(leaves, buds or flowers) were ground in 1 ml distilled
sterile water with 0.1% βME in a plastic bag lined with
cheesecloth using a tissue-X homogenizer (Bioreba AG). The
resulting sap was transferred to a microcentrifuge tube with
a pasteur pipette and centrifuged briefly to sediment leaf
debris. Fifty μl supernatant were transferred to 450 μl 10%
Triton X-100, heated to 65°C for 10 minutes and then placed
on ice. A 10 μl sample was removed and denatured by the
addition of 2 μl 40 mM MeHgOH. Samples were left at room
temperature for 10 minutes and then treated with 1 μl 260 mM
βME. A reverse transcription cocktail was then added, giving
the same reaction conditions as set out in section 2.3.1, in
a final volume of 20 μl. The reaction was incubated at 37°C
for 1 hour. It was primed with random hexamers.
The reaction was brought to 50 μl by the addition of 17 μl sterile water, 2 μl 10 mM dNTPs, 2 μl of each primer (2 μM final concentration), 5 μl 10X Taq buffer (Stratagene) and 0.5 μl Taq DNA polymerase (Stratagene). The sample was heated to 95°C for 2 minutes and cycled through 35 cycles of: denaturation, 1 minute at 95°C; primer annealing, 1 minute at 42°C; DNA synthesis, 1 minute at 72°C. There was a final extension for 4 minutes at 72°C. The products of the reaction were analyzed by agarose gel electrophoresis and stained with ethidium bromide. The presence of PDV was indicated by the appearance of a reaction product corresponding to its specific primer pair. In general, each sample was assayed at least twice and every tenth sample was a template-negative control.

2.4.0 Preparation of a defective interfering particle

2.4.1 Production of a snapback-type DI particle by PCR

One of the RACE clones (#18) containing the 5' end sequence of RNA1 and a Pvu I site at position 267 was ligated to pPDV33 (section 2.3.11) using the PvuI site; this was done as follows. Clone #18 was digested with Pvu I and BamHI and a 265 bp fragment isolated from an agarose gel using Qiaex beads. Plasmid pPDV33 was digested with BglII and PvuI and a 1030 bp fragment isolated from an agarose gel. The concentrations of these fragments was determined by spectrophotometry and they were mixed in a 1:1:3 molar ratio
with pBluescript SK+ linearized with BamHI, and incubated with T4 DNA ligase (Gibco/BRL) in ligase buffer (Gibco/BRL) overnight at room temperature. The ligation products were transformed into E. coli DH5α and selection was carried out by PCR using the M13 universal and reverse primers (section 2.3.6) to search for clones containing an insert 1.3 kb in size. This clone would contain the 5'-end of RNA1 and the sequence up to position 1295; it was called pPDV1300.

Three primers were synthesized to construct a snapback type DI particle by PCR. These had the following sequences:

DI1: TAAGGATCCTAATACGACTCCTAGTAGTTTACGAACGTGGTTGTTC
DI2: TAAGGATCCGCCGTTTTACGAACGTGGTTGTTC
DI3: ATGGACAACGCGCTGAT

DI1 features a BamHI site (GGATCC), the T7 promoter (underlined) and the first 22 bases of PDV RNA1 (on pPDV1300). DI2 also has a BamHI site, to facilitate cloning, a unique SstII site (CCGCGG) to linearise the final construct before transcription and the first 22 bases of pPDV1300. DI3 is complementary to a region beyond a ClaI (BspDI) site on pPDV1300 about 1 kb downstream from DI1/2.

Primer pairs DI1 & DI3 and DI2 & DI3 were used to amplify pPDV1300 in separate PCR reactions. The PCR reaction was catalyzed by Taq DNA polymerase with the buffer conditions recommended by the manufacturer (Stratagene) using...
each primer at a concentration of 20 μM. The optimal annealing temperature was determined empirically to be 48°C and the following conditions were used for 35 cycles in a thermocycler: denature 30 seconds at 94°C; anneal 40 seconds at 48°C; extend 1 minute at 72°C. After PCR, the products were precipitated in ethanol, the pellet dissolved in 9 μl NEB buffer 3 (New England Biolabs) and digested with 5 U BspDI and 5 U BamHI (New England Biolabs) for 1 hour at 37°C. The entire digest was loaded onto a 1% agarose gel and the fragments separated electrophoretically. The desired band was approximately 940 bp in length for each reaction. The band generated by DI1 & DI3 is 15 bases longer, but in practise this could not be distinguished from the product of DI2 & DI3.

The 940 bp bands were extracted from the gel and purified with Qiaex beads (Qiagen). The DNA concentrations were determined spectrophotometrically and the fragments were mixed in a 1:1:3 molar ratio with pUC18 linearized with BamHI and treated with CIP. The mixture was incubated overnight at room temperature with 1 U T4 DNA ligase in ligase buffer (Gibco/ BRL). Competent E.coli DH5α were transformed with the reaction on the following day. The insert size of white colonies was determined by PCR screening using the M13 universal and reverse primers on pUC18. A band approximately 2.1 kb in size (1.9 kb insert plus 200 bp polylinker) on an agarose gel would indicate a potential DI template for
transcription. Since three different ligation products would give an insert of this size, all positive clones also had to be screened by digestion with SstII. Only clones with a single SstII site would be correct. There is a unique SstII site in the DI2 primer; pUC18 is not cut by this enzyme.

2.4.2 Preparation of a snapback-type DI particle with synthetic oligonucleotides.

An alternate strategy for the production of DI particles, which required no PCR, was also devised. This strategy relies on the presence of a BspHI site 40 bp in from the 5’ end of pPDV1300. Four oligonucleotides were synthesized using a Beckman 1000M DNA synthesizer, Table 1. Oligonucleotides 1 & 2 are designed so that when they anneal they produce a HindIII compatible end to facilitate cloning and the T7 promoter immediately adjacent to the first 40 bases of pPDV1300, ending in a BspHI compatible end.

Oligonucleotides 3 & 4 are designed so that when they anneal they produce a KpnI compatible end for cloning, a unique XmaI site to linearize the construct before transcription and the complement of the first 40 bases of pPDV1300 ending in a BspHI compatible end.

After the synthesis, these oligonucleotides were dissolved in 100 µl of distilled water and their concentrations determined by spectrophotometry. Equal molar amounts of oligo1 & oligo2 and oligo3 & oligo4 were mixed,
Table 1. Oligonucleotides used to construct templates for snapback and deletion-type DI particles. Oligos #1 and #2 align to form a cassette with HindIII and BspHI ends and with the T7 promoter directly adjacent to the 5' end sequence of PDV RNA1. Oligos #3 and #4 align to form a cassette with KpnI and BspHI ends and have an XmaI site adjacent to the KpnI site. When a palindromic cDNA fragment, derived from the 5' end of RNA1, is ligated to these cassettes via their BspHI ends a palindromic sequence results which, when transcribed with T7 RNA polymerase, gives rise to a snapback DI RNA.

<table>
<thead>
<tr>
<th>Oligo</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AGCTTAATACGACTCACTATAGGTTTTACGAACGTGGTTGTTCGTATTTTAAATCAAT</td>
</tr>
<tr>
<td></td>
<td>ATTATGCTGAGTGATATCCAAAATGCTTGCACCAACAAGCATATAAAATTTAGTTAGTAC</td>
</tr>
<tr>
<td>2</td>
<td>CATGATTGATTTAAATACGAACCAACGTTTACGAACGTGGTTGTTCGTATTTTAAATCAAT</td>
</tr>
<tr>
<td></td>
<td>ATTATGCTGAGTGATATCCAAAATGCTTGCACCAACAAGCATATAAAATTTAGTTAGTAC</td>
</tr>
<tr>
<td>3</td>
<td>CATGATTGATTTAAATACGAACCAACGTTTACGAACGTGGTTGTTCGTATTTTAAATCAAT</td>
</tr>
<tr>
<td></td>
<td>ATTATGCTGAGTGATATCCAAAATGCTTGCACCAACAAGCATATAAAATTTAGTTAGTAC</td>
</tr>
<tr>
<td>4</td>
<td>CATGATTGATTTAAATACGAACCAACGTTTACGAACGTGGTTGTTCGTATTTTAAATCAAT</td>
</tr>
<tr>
<td></td>
<td>ATTATGCTGAGTGATATCCAAAATGCTTGCACCAACAAGCATATAAAATTTAGTTAGTAC</td>
</tr>
</tbody>
</table>

(D)
overlayed with parafilm oil, heated to 80°C for 10 minutes and allowed to cool to room temperature over a period of 2 hours. These were ligated onto the ends of pUC18 by mixing in a 3:3:1 molar ratio with pUC18, previously linearized with HindIII and KpnI, and incubating with 1 U T4 DNA ligase in ligase buffer (Gibco/BRL) for 4 hours at room temperature. The ligation products were separated from free oligonucleotides by electrophoresis and the 2.7 kb band was extracted from the gel using Qiaex beads (Qiagen). The concentration of the resulting products was determined by spectrophotometry.

The bulk of the genetic material for the DI particle was made from pPDV1300 by digesting the plasmid with PvuI and BspHI. This released a 225 bp fragment which was gel purified using Qiaex beads. The concentration of this DNA was determined and the fragment was mixed in a 10:1 molar ratio with the prepared pUC18 (above) and incubated overnight at room temperature with 2 U T4 DNA ligase in ligase buffer (Gibco/BRL). Although the prepared pUC18 vector has compatible BspHI ends, neither of these is phosphorylated and thus circularization without an insert is prevented. Also, the PvuI fragments can join either via the PvuI or the BspHI ends but since they can only ligate to the vector if they have two BspHI ends, and since either orientation will produce the same clone, almost all positive transformants would be correct.
2.4.3 Preparation of a deletion type-DI particle

A template for transcribing a deletion-type DI RNA was assembled from two existing clones, pPDV33 (2.3.11) and a RACE clone containing the 3' end of RNA1 (pRACE32) (see Figure 1). The former was digested with EcoRV and BamHI to remove approximately 460 bp from its 3' end. pRACE32 was digested with HincII and BamHI and the 150 bp fragment, representing the 3'-end of RNA1 was gel purified and ligated into prepared pPDV33 described above. Transformants were screened for insert size, and a correct clone was identified and amplified. This clone was digested with BamHI and BspHI and cloned into pUC18-T7.

Plasmid pUC18-T7 was prepared by digesting pUC18 with BamHI and HindIII and ligating oligonucleotides 1/2 (see Table 1) into the HindIII site. The resulting plasmid pPDVdil was sequenced to confirm its structure. A 1 µg preparation was digested with BamHI and transcribed using T7 RNA polymerase (Ribomax kit: Promega). After the reaction, DNA was removed by digestion with DNase I and the RNA recovered by phenol extraction and ethanol precipitation.

2.4.4 Replication of a DI particle in vivo

Two µg of purified DI RNA in TE (10 mM Tris- HCl, pH 8.0; 1 mM EDTA) were mechanically inoculated onto pumpkin cotyledons dusted with carborundum (C. maxima cv. Small Sugar). These had been infected with PDV 1 week earlier.
Figure 1. The structure of pPDVdi1. The construct was produced by digesting pPDV33 (yellow) with EcoRV and BspHI and a full length RACE clone (pRACE32, black) containing the 3'-end of PDV RNA1, with BamHI and HincII. These clones were ligated via their blunt ends and ligated into pUC18, which contained the T7 promoter cassette described in Table 1, via the BspHI and BamHI sites.
Healthy control plants were also inoculated with DI RNA and some PDV-infected plants were mock-inoculated with TE buffer. Plants were allowed to grow for a period of 3 days and harvested. Total nucleic acid was extracted (section 2.1.5), separated by electrophoresis on a nondenaturing agarose gel and transferred to a nylon membrane by a capillary blot. The Northern blot was probed with a radiolabelled cDNA probe, prepared by labelling a PvuI/NcoI fragment of pPDV33 with $^{32}$P, using random primer labelling (section 2.3.7). The nylon membrane was autoradiographed to visualize bands.
3.0 RESULTS

3.1.0 Virus Purification

The virus purification procedure of Fulton (1959) yielded virus of adequate purity for many applications. This procedure was used to provide antigen for TAS-ELISAs which required purified PDV and for the detection of the PDV coat protein by western blotting. Virions prepared by this method were found by SDS PAGE to be contaminated by other proteins. Experiments which required purer virions were done with virus which had been purified further by linear sucrose density gradient ultracentrifugation, which eliminated most of the plant contaminants (see Figure 2). Fractions taken from the sucrose gradient after ultracentrifugation were analyzed by SDS PAGE to assess the effect of this step on virus purity (Figure 2A), and to determine the optimal centrifugation time. Figure 2A shows the gradient ultracentrifugation run for 2 hours. When this time was increased to 2.5 hours, the virus moved to the center of the gradient and was separated from protein contaminants, indicated by a single band in the 25 kDa region in lane 3, Figure 2B. The M_r of the coat protein of ilarviruses has been reported to be typically 25 kDa (Fulton, 1975) and the presence of a band in this region, absent in negative control lanes, was used as an indicator of the presence of PDV.

The yield of purified virus, collected by ultra-
Figure 2. Denaturing polyacrylamide electrophoresis gels (SDS PAGE) showing virus purity after ultracentrifugation through a linear sucrose gradient. The gels were stained with Coomassie brilliant blue R-250, molecular weight markers are given in kDa. A. Gradient fraction analysed after 2 hours of ultracentrifugation; lane 1: molecular size standards (dalton VII set: Sigma); lane 2: sample loaded onto sucrose gradient (pre-gradient fraction); lane 3: upper part of gradient; lane 4: central band in gradient; lane 5: lower part of gradient; (lane 6: empty); lane 7: preparation from uninfected leaves (pre-gradient fraction cf. lane 2).

B. Ultracentrifugation (sucrose gradient) run for 2.5 hours; lane 1: pre-gradient fraction; lane 2: upper part of gradient; lane 3: central peak in gradient; (lane 4: empty); lane 5: healthy preparation (cf. lane 7 in A); lane 6: molecular size standards (dalton VII set: Sigma). The $M_r$ of ilarvirus coat protein is reported to be typically 25 kDa.

Samples were not from the same virus preparation and, due variations in the preparations, the same amount was not loaded onto each gel.
centrifugation of the pooled sucrose gradient peaks, was determined by spectrophotometry for each preparation based on the reported extinction coefficient for PDV of 1.57 at 260 nm (Halk & Fulton 1978). Virus recovery was found to be typically 25 μg virus/g fresh weight leaf tissue (approximately 2 mg total per preparation).

3.1.1 Viral RNA separation

Extraction of genomic ssRNA directly from isolated virus particles using heated phenol was effective and ensured that the viral RNA was almost free of contaminating plant rRNAs, since ribosomes were mostly eliminated during the virus purification procedure. Genomic PDV RNA was analyzed by MeHgOH agarose gel electrophoresis to determine the sizes of the three RNA species (Figure 3A). The sizes were: RNA1: 3.6 kb; RNA2: 2.8 kb; RNA3: 2.2 kb. The subgenomic RNA4 has a reported size of 0.88 kb (Bachman et al. 1994) and is clearly visible in Figure 3A.

Replicative form dsRNA was analyzed by non-denaturing agarose gel electrophoresis using CMV dsRNA as molecular size marker (Figure 3B). The dsRNA was used in Northern blots to identify the origin of cDNA fragments. DsRNA preparations were frequently contaminated with rRNA and tRNA but this could be reduced by the addition of RNase T1 to the sample or electrophoresis. This simplified the identification of dsRNA bands. Contamination by plant DNA was easily eliminated by
Figure 3A. Determination of the sizes of the PDV RNA species (lanes 1 & 2) by denaturing agarose gel electrophoresis in MeHgOH. The gel was stained with ethidium bromide. The molecular weight standards (lane 3, Gibco/BRL) are given in kb. The sizes of the RNAs were determined to be: RNA1: 3.3 kb; RNA2: 2.6 kb; RNA3: 1.9 kb. RNA4 (0.88 kb) is visible at the bottom of the lane.

B. Confirmation of the presence of replicative form dsRNA after extraction from infected cherry leaves (lane 2). The molecular size standard is CMV dsRNA and sizes are shown in kb (lane 1). The gel was stained with ethidium bromide.
by allowing RNase A to hydrolyse single-stranded RNA after
digestion with DNase1.

3.2.0 Antibody Production

The sera from the tail-bleeds of two immunized mice were
assayed for anti-PDV antibodies. The results of a TAS-ELISA,
expressed as PDV/healthy, were 2.246/0.876 and 2.239/0.891
for a 1:3000 dilutions of the sera, indicating that both mice
had an immune response against the virus. The fusion yielded
a single stable hybridoma line, called PDA-3C, although two
other hybridomas were also identified that produced anti-PDV
antibodies. However, only the PDA-3C hybridoma line survived
in vitro and it was used in all subsequent serological
assays.

Serological testing revealed that this antibody was of
the IgG1 subclass. The HAP purified stock had a protein
concentration of 2.5 mg/ml and a dilution endpoint of 1:15000
by TAS-ELISA. Routine assays were carried out with the
antibody at a dilution of 1:1000 or 2.5 μg/ml. To verify
that PDA-3C was reacting with the coat protein of PDV, it was
tested in an immunoblot (Figure 4). The antibody binds to a
protein with a M_r of approximately 25 kDa, corresponding to
the size of the coat protein typically reported for members
of the ilarvirus group.

PDA-3C was evaluated in a TAS-ELISA to detect PDV in the
flowers and leaves of a PDV-infected sweet cherry and the
Figure 4. Recognition of a 25 kDa band by PDA-3C in a Western blot. The SDS-PAGE used to make this blot was loaded with a single wide-tooth comb. Molecular weight markers were run but are not shown in the figure; however their positions and sizes are indicated in kDa. The gel was loaded with PDV purified as described in section 2.1.2.
P. mahaleb stock tree (Table 2). The assay could routinely detect PDV during April and May giving absorbance values above the positive-negative threshold for infected trees, but was no longer reliable by late June. The positive-negative threshold in Table 2 was set at $x_n + 4S$, (the mean of the healthy controls plus four standard deviation units (Sutula et al. 1986)). When trees infected with PNRSV were assayed by TAS-ELISA using PDA-3C, the absorbance values were below the threshold established from uninfected trees (Table 2). Thus, PDA-3C did not cross-react with PNRSV in this assay. There are no other known ilarviruses which naturally infect sweet cherry (Németh, 1986).

PDA-3C was also tested in a TAS-ELISA against two isolates of PDV giving particularly severe symptoms, obtained from the virus collection in Wenatchee, WA. The results of this assay, in Table 2, show that the antibody was able to recognize these strains of PDV.

3.3.0 Primer pairs #1 and #2 in RT-PCR

Once a protocol for the amplification of purified PDV RNA by RT-PCR had been established, two primer pairs (section 2.3.15) were tested for their ability to detect PDV in the leaves of the P. mahaleb PDV stock tree, a healthy control sweet cherry tree and a tree infected with PNRSV. The RT-PCR procedure gave positive results with both primer pairs (Figure 5) for the PDV infected tree only. Amplification
Table 2. TAS-ELISA results using monoclonal antibody PDA-3C to differentiate between PDV and PNRSV in infected sweet cherry and *P. mahaleb*. Three samples were assayed in each case. Absorbance measurements were made following a 2 hour substrate incubation.

<table>
<thead>
<tr>
<th>Sample</th>
<th>PDV-infected</th>
<th>PNRSV-infected</th>
<th>Healthy</th>
<th>Buffer blank</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. avium</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Summerland</td>
<td>Flower</td>
<td>0.428±0.041</td>
<td>0.073±0.021</td>
<td>0.066</td>
</tr>
<tr>
<td></td>
<td>Leaf</td>
<td>0.222±0.024</td>
<td>0.071±0.009</td>
<td></td>
</tr>
<tr>
<td><em>P. mahaleb</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Summerland</td>
<td>Flower</td>
<td>0.991±0.048</td>
<td>ND¹</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Leaf</td>
<td>0.460±0.076</td>
<td>0.065±0.003</td>
<td>0.054</td>
</tr>
<tr>
<td><em>P. avium</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wenatchee²</td>
<td>Leaf</td>
<td>0.751±0.027</td>
<td>ND</td>
<td>0.088</td>
</tr>
<tr>
<td></td>
<td>Leaf</td>
<td>0.666±0.044</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

¹ND = not determined
²Two isolates were assayed
Figure 5. Results of RT-PCR assay of the *P. mahaleb* positive control tree, a healthy sweet cherry control tree and a sweet cherry infected with PNRSV. The gel is stained with ethidium bromide. Lane 1: molecular size standards (100 bp ladder: Gibco/BRL), sizes indicated in bp; lanes 2 & 3: template-negative control for primer pairs #1 & #2 respectively; lane 4: the *P. mahaleb* tree with pair #1; lane 5: healthy control for pair #1; lane 6: the *P. mahaleb* tree with pair #2; lane 7: healthy control with pair #2; lane 8: PNRSV infected tree with pair #1; lane 9: PNRSV infected tree with pair #2.
failed in the healthy tree and furthermore, neither pair amplified RNA from PNRSV. Thus, either pair could be used to detect PDV in infected cherry.

3.3.1 Detection of PDV by RT-PCR in sweet cherry

Since RT-PCR is currently one of the most sensitive method available for PDV detection, it was used to determine the PDV-infection status of sweet cherry trees which were later assayed by TAS-ELISA and bioassay (section 3.4.2). Fifty-five trees were originally selected for a survey designed to establish a reliable TAS-ELISA assay, and these were assayed for the presence of PDV by RT-PCR in late winter using unopened buds and in spring using flowers and leaves. The results of the RT-PCR using leaves are shown in Figure 6; the presence of a 197 bp band indicates the presence of PDV in a tree. It was found that leaves were the most convenient tissue to work with in terms of ease of processing and hence lower chance of sample cross contamination.

3.4.0 Results of the field survey

3.4.1 Identification of PDV-infected trees

Of the 55 trees originally tested by RT-PCR, 40 trees were used in a field survey to establish a reliable TAS-ELISA (Table 3). The results of the RT-PCR assay performed on the symptomatic trees indicated the PDV infection status of these samples are summarized in Table 4. The 15 symptomless trees,
Figure 6. Agarose gel, stained with ethidium bromide, showing RT-PCR results. Leaves were assayed for the presence of PDV in the spring, shortly after petal-drop. Some of these trees were used to set confidence limits for the TAS-ELISA. This group was assayed with primer pair #1, and the presence of a 179 bp band indicates the presence of PDV. The molecular size standards (100 bp ladder: Gibco/BRL) are given in bp. Lanes are labelled with the tree location, each lane represents one tree.
Table 3. Results of the PDV TAS-ELISA, RT-PCR and bioassay and the PNRSV TAS-ELISA performed on forty cherry trees at the Summerland Research Centre. Most of the trees had symptoms indicating a viral infection. TAS-ELISA results are indicated by the number of infected leaves out of a possible six.

<table>
<thead>
<tr>
<th>Tree</th>
<th>Symptoms</th>
<th>TAS-ELISA</th>
<th>Bio-assay</th>
<th>RT-PCR</th>
<th>PNRSV</th>
</tr>
</thead>
<tbody>
<tr>
<td>13S 22/8</td>
<td>None</td>
<td>5/6 +</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>13S 24/28</td>
<td>None</td>
<td>0/6 -</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>13S 39/51</td>
<td>None</td>
<td>6/6 +</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>2N 36/26</td>
<td>Shot holes</td>
<td>6/6 +</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>2N 34/23</td>
<td>Shot holes</td>
<td>0/6 -</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>11-14</td>
<td>Shot holes</td>
<td>0/6 -</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>SP 4/3</td>
<td>Shot holes</td>
<td>6/6 +</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>A 2/1</td>
<td>Shot holes, necrosis</td>
<td>2/6 +</td>
<td>+</td>
<td>+</td>
<td>ND*</td>
</tr>
<tr>
<td>A 2/5</td>
<td>Little cherry disease</td>
<td>5/6 +</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>A 9/34</td>
<td>None</td>
<td>0/6 -</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>A 9/41</td>
<td>Rugose</td>
<td>6/6 +</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>A 9/71</td>
<td>Shot holes</td>
<td>6/6 +</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>A 11/3</td>
<td>Red leaves</td>
<td>6/6 +</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>A 20/1</td>
<td>Red leaves</td>
<td>6/6 +</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>A 20/2</td>
<td>Red leaves</td>
<td>5/6 +</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>A 20/3</td>
<td>Red leaves</td>
<td>6/6 +</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>A 20/4</td>
<td>Red leaves</td>
<td>6/6 +</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>B 4/2</td>
<td>Shot holes, brown patches</td>
<td>6/6 +</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>B 4/3</td>
<td>Twisted leaf, necrotic midvein</td>
<td>0/6 -</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B 4/27</td>
<td>Shot holes, enations</td>
<td>6/6 +</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>B 4/52</td>
<td>Twisted leaf, dieback, ringspots</td>
<td>6/6 +</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>B 5/5</td>
<td>White shoulders</td>
<td>0/6 -</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B 6/24</td>
<td>Mottle, bumpy fruit</td>
<td>6/6 +</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>B 6/25</td>
<td>Mottle, shot holes</td>
<td>6/6 +</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>B 8/30</td>
<td>Shot holes</td>
<td>6/6 +</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>B 8/34</td>
<td>Shot holes</td>
<td>6/6 +</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>B 8/38</td>
<td>Red leaves</td>
<td>4/6 +</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>B 9/22</td>
<td>Shot holes</td>
<td>6/6 +</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>B 9/30</td>
<td>Shot holes</td>
<td>4/6 +</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Code</td>
<td>Description</td>
<td>Value</td>
<td>±</td>
<td>-</td>
<td>±</td>
</tr>
<tr>
<td>--------</td>
<td>------------------------------</td>
<td>-------</td>
<td>----</td>
<td>----</td>
<td>---</td>
</tr>
<tr>
<td>B 9/45</td>
<td>Shot holes</td>
<td>0/6</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>B 9/47</td>
<td>Shot holes</td>
<td>6/6</td>
<td>+</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>B 9/48</td>
<td>None</td>
<td>6/6</td>
<td>+</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>B 9/49</td>
<td>Shot holes, mottle</td>
<td>0/6</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>B 9/50</td>
<td>None</td>
<td>6/6</td>
<td>+</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>B 9/51</td>
<td>None</td>
<td>6/6</td>
<td>+</td>
<td>+</td>
<td>ND</td>
</tr>
<tr>
<td>B 13/12</td>
<td>Short stem</td>
<td>5/6</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>B 13/22</td>
<td>Shot holes, mottle, yellows</td>
<td>6/6</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>SP 2/18</td>
<td>Shot holes</td>
<td>0/6</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>B 5/2</td>
<td>Shot holes</td>
<td>1/6</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>A 16/1</td>
<td>None</td>
<td>0/6</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

*ND = not determined*
to be used as negative controls, were determined to be PDV-free by RT-PCR and ilarvirus-free by the 'Shirofugen' bioassay. PDV and PNRSV are indistinguishable by indexing on 'Shirofugen', necessitating further investigation of five trees that tested positive by the bioassay but negative for PDV by RT-PCR. These trees were found to be infected with PNRSV in a separate TAS-ELISA (Table 4).

3.4.2 Detection of PDV by TAS-ELISA

The results of the TAS-ELISA on the group of 40 symptomatic trees to determine the number of leaves which tested positive for PDV by TAS-ELISA out of a possible six for each tree are summarized (Table 5). Average ELISA values, as well as maximum and minimum values for each set, are expressed relative to the threshold to normalize them for each plate. The proportion of leaves that were infected was not the same for every infected tree ($\chi^2 = 87.7$, df = 30, $P<0.001$). PDV was detected in ≥ 4/6 leaves by TAS-ELISA in 29 of the 31 infected trees. Thus, most infected trees would be identified if the number of leaves to be tested was chosen so that trees with two-thirds or more of their leaves positive by TAS-ELISA had a high probability, ie. 99%, of being identified. Using the formula $n = \log(1-P)/\log(1-Pr)$ where $n$ is the number of leaves to be tested, $P$ is the desired probability of detection (0.99) and $Pr$ is the proportion of infected leaves (0.667), it was determined
Table 4. Summary of the results of the RT-PCR, bioassay and PNRSV TAS-ELISA carried out on 40 symptomatic test-trees and 15 symptomless negative control trees to establish the incidence of PDV, ilarviruses and PNRSV respectively.

<table>
<thead>
<tr>
<th>Symptomatic trees</th>
<th>Assay Results</th>
<th>Number of trees</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RT-PCR</td>
<td>Bioassay</td>
</tr>
<tr>
<td>Symptomatic trees</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Symptomless trees</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*ND = not determined
Table 5. Summary of the TAS-ELISA results of trees infected with PDV; six leaves were assayed from each tree in duplicate assays. To normalize the data between plates mean, minimum and maximum values are expressed as a ratio of absorbance and threshold for each plate.

<table>
<thead>
<tr>
<th># Positive leaves per tree (out of 6)</th>
<th># Trees</th>
<th>ELISA Results (Sample value/threshold)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>1.885</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>1.273</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>1.973</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>1.508</td>
</tr>
<tr>
<td>6</td>
<td>23</td>
<td>1.931</td>
</tr>
<tr>
<td>Total:</td>
<td>31</td>
<td></td>
</tr>
</tbody>
</table>
that four leaves from each tree must be tested in order to achieve this level of accuracy in the TAS-ELISA. Trees with more than two-thirds of their leaves infected would have a higher probability of detection while trees with fewer infected leaves would have a lower probability of detection.

3.5.0 Alternate trapping antibodies.

The F(ab')₂ fragments produced by pepsin digestion of PDA-3C were analyzed by SDS PAGE (Figure 7). Samples reduced by βME and unreduced samples were analysed. The presence of a band with an M_r of 110 kDa, indicated the presence of unreduced F(ab')₂ fragments. These were used in place of the PVAS-290 rabbit serum to trap PDV in a TAS-ELISA.

Chicken IgY antibodies were also assessed for their ability to trap PDV in a TAS-ELISA and monoclonal PDA-3C was used with alkaline phosphatase conjugated PDA-3C in a DAS-ELISA. The results of these assays are summarised in Table 6.
Figure 7. Results of SDS PAGE used to analyse F(ab')₂ fragments. Digestion of monoclonal PDA-3C with pepsin yielded a band with Mr of 110 kDa, resistant to digestion after 10 hours (lane 1) and 24 hours (lane 3). This band is eliminated by reduction of the sample with βME before loading (lane 2: 10 h sample; lane 4: 24 h sample). The 110 kDa band is absent in lane 5 (unreduced PDA-3C, IgG₁) and in lane 6 (reduced PDA-3C). This band is the correct size reported for F(ab')₂ fragments (Harlow & Lane 1988). Pepsin used for the digest was run in lane 7. This 7% SDS-polyacrylamide gel was stained with silver nitrate. Molecular weight markers (High MW set: Sigma, lane 8) are shown in kDa.
Table 6. Results of ELISAs using different trapping and detecting antibodies. The numbers represent ELISA values ($A_{405}-A_{620}$) of the optimal dilution scheme for each assay format.

<table>
<thead>
<tr>
<th>Trapping antibody</th>
<th>Detecting antibody</th>
<th>ELSIA value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PDA-3C-AP(^4) (1:200)(^2)</td>
<td>PDA-3C (1:1000) with AP-conjugate(^3)</td>
</tr>
<tr>
<td>1) PDA-3C (1:400)</td>
<td>PDV(^4): 2.183</td>
<td>ND(^6)</td>
</tr>
<tr>
<td></td>
<td>Healthy(^5): 1.482</td>
<td></td>
</tr>
<tr>
<td>2) F(ab')(_2) (1:800)</td>
<td></td>
<td>PDV: 0.621</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Healthy: 0.261</td>
</tr>
<tr>
<td>3) ATCC PVAS 290, (1:2000)</td>
<td></td>
<td>PDV: 0.672</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Healthy: 0.078</td>
</tr>
<tr>
<td>4) IgY (1:625)</td>
<td></td>
<td>PDV: 0.210</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Healthy: 0.089</td>
</tr>
</tbody>
</table>

\(^1\) PDA-3C-AP = alkaline phosphatase-linked PDA-3C, in a DAS-ELISA.
\(^2\) Concentration giving optimal results.
\(^3\) AP conjugate = sheep anti-mouse (F\(_c\) region) alkaline phosphatase-linked polyclonal (KPL) for detecting Ab 2.
\(^4\) PDV sample was a PDV-infected sweet cherry leaf, except for IgY assay, where an infected cucumber cotyledon was used.
\(^5\) Healthy sample was an uninfected sweet cherry leaf, except for IgY assay, where a healthy cucumber cotyledon was used.
\(^6\) ND = not done.
3.6.0 The partial sequence of PDV RNA3

The partial sequence for PDV RNA3 (from clones PDV3a and PDV3b) and their alignment to the reported sequence of RNA3 (Bachman et al. 1994) is shown in Figure 8. The position of the two primer pairs used for RT-PCR and the ORFs are shown. There are seven differences indicated at the nucleotide level. The predicted ORF1 & ORF2 products of the published sequence were compared to the translation products of PDV3a and PDV3b (results not shown). There were three differences at the amino acid level: leucine to valine (position 906 in the published nucleotide sequence of RNA3, in the movement protein); asparagine to lysine (position 1584, coat protein); proline to arginine (position 1590, coat protein). For amino acid comparison, the sequence of the RNA3 clones was kept in-frame with the published sequence if nucleotide insertions or deletions occurred, such as at position 905.

3.7.0 The complete nucleotide sequence of PDV RNA1

A total of eight clones was sequenced in both directions to obtain the complete sequence of RNA1. A map of the positions of these clones is shown in Figure 9. A further 12 RACE clones were also sequenced to obtain the sequence of the ends of the RNA (clones not shown in Figure 9).

The complete nucleotide sequence of PDV RNA1 and its putative translation product are shown in Figure 10. The
...
Figure 8. The published sequence (top) of PDV RNA3 (Bachman et al. 1994) aligned with two fragments of PDV RNA3 sequence obtained (position 545 to 1323 and 1444 to 1819). Mismatches between the sequences are indicated by (-) or by the nucleotide. The two open reading frames (position 260 to 1141 and 1214 to 1868) of the published sequence are shown in capitals, UTRs are in lowercase letters. The positions of PCR primer pairs #1 (position 844 and 1109) and #2 (position 1296 and 1456) are underlined. Initiation and termination codons are shown in boldface.
Figure 9. Map of RNA1 (---) indicating the relative positions of nine clones, eight of which were sequenced completely, to obtain the sequence of RNA1. The RACE clones are not shown. All numbers are in bp measured from the 5'-terminus of RNA1 unless otherwise indicated.
Figure 10. The complete nucleotide sequence of PDV RNA1 and its putative translation product. Numbers indicate nucleotide or amino acid position. * Indicates in-frame stop codons in the 3’-UTR.
sequence is 3374 nucleotides in length and could encode a single protein of 1055 amino acids with a calculated molecular mass of 118.9 kDa. The length of the RNA sequence is in good agreement with the predicted length of 3.4 kb, estimated by denaturing agarose gel electrophoresis of total genomic PDV RNA (Figure 3). The calculated size of the protein is similar to that of AMV (128 kDa) and CiLRV 118.3 kDa). The initiation codon for the 118.9 kDa protein is at position 39 and the first in-frame termination codon occurs at 3202. There are no other ORFs longer than 93 amino acids on the RNA.

The 3'-UTR of RNA1 is 171 bases long and shares extensive sequence homology with other ilar- and alfamovirus 3'-UTRs (Figure 11A), including five U/AUGC motifs which appear in the 3'-UTRs of all known ilarvirus sequences. In each AMV RNA these AUGC motifs flank short sequences which can form stem-loop structures (Houser-Scott et al. 1994). Although the intervening sequences are not strictly conserved between PDV and AMV RNA1, the relative positions of the AUGC motifs are in good agreement, and the 3'-end of PDV RNA1 can form essentially the same structure as the 3'-end of AMV RNA1 (Figure 11B).
Figure 11. Comparison of the 3'-ends of some ilarvirus RNAs and AMV RNA1. A. The conserved U/AUGC motifs are underlined to demonstrate potential relationships. These motifs flank conserved sequences which could form stem-loop structures. B. The folding of the 3' end of PDV RNA1 is very similar to the 3' stem-loop structure proposed by Koper-Zwartoff & Bol (1980) for the 3' end of AMV.
3.7.1. Phylogenetic relationships of PDV to other Bromoviridae, based on RNA1

The phylogenetic relationship of members of the Bromoviridae and RBDV is predicted on the basis of the nucleic acid sequence of their RNA1s and of their translation products (Figure 12A). The numbers at the forks indicate the number of times the group to the right of the fork occurred out of a possible 100 trees. This pairing was reproducible and occurred regardless of whether the RNA or the amino acid sequence was analysed. Since there were two clear blocks of homology in the PILEUP alignments of the six sequences, the SEQBOOT analysis was repeated using either the N-terminal or helicase-like domain (Candresse et al. 1990) blocks of homology individually. The resulting phylogeny was the same as in Figure 12A. A similar phylogeny results from parsimony analyses of bootstrap replicates made with RNA3 and ORF3a (the putative movement protein; Figure 12B).

3.8.0 Replication of a DI RNA

Figure 13A shows the DI RNA (transcription product) on a non-denaturing agarose gel. The DI RNA is approximately 1 kb in size and was transcribed from pPDVdil by T7 RNA polymerase. Figure 13B shows results of a Northern blot, using total RNA isolated from plants inoculated with DI RNA in the presence and absence of PDV as the target and a PvuI/NcoI fragment of pPDV33 as the probe. Bands which
Figure 12. Phylogenetic relationships inferred by parsimony analysis with Phylib 3.4 using PROTPARS and DNAPARS of 100 Bootstrap replicates generated by SEQBOOT. Nucleic acid and protein alignments were generated by PILEUP (GCG) and edited using XESEE.

A. Relationships between RNA1 and ORF1 of at least one member of each genera of the Bromoviridae and RBDV. RBDV RNA1 encodes a polyprotein and only the C-terminal region was used in the analyses. Fork numbers indicate the number of times the virus group to the right of that fork occurred in a total of 100 trees.

B. Relationships between RNA3 and movement proteins (ORF3a) using the same analyses as in A.
Figure 13A. Agarose gel electrophoresis of the product of *in vitro* transcription of pDI1. B: Northern blot analysis of plant total RNA, probed with a PvuI/NcoI fragment of pPDV33 (complementary to the 5′-end of RNA1). Lane 1: DI RNA transcript (positive control); lane 2: RNA from plant inoculated with PDV and DI RNA; lane 3: RNA from plant inoculated with PDV only; lane 4: RNA from plant inoculated with DI only; lane 5: RNA from uninoculated plant (negative control). Plants (pumkins) were inoculated with DI RNA 1 week after they had been inoculated with PDV and leaves were harvested 3 days later.
hybridized are viral RNA1 replicating in the leaves. The DI RNA failed to replicate in vivo using PDV infected pumpkins as a herbaceous model system and there were no bands at approximately 1 kb (the position expected for the DI RNA) which hybridized to the probe. Furthermore the presence of inoculated DI RNA did not appear to influence the copy number of PDV RNA1.
4.1.0 Virus isolation and nucleic acid analysis

An existing isolation technique, developed by Fulton (1959), was modified to include a sedimentation velocity ultracentrifugation step. This removed many of the protein contaminants present in earlier fractions and resulted in a single major band corresponding to the coat protein of PDV when analyzed by SDS PAGE. This purified virus was used to immunize animals to produce polyclonal and monoclonal antibodies. The virus preparation was also found to be relatively free of plant ribosomes and was thus suitable for isolating single-stranded genomic viral RNA which was relatively free of plant rRNA. The viral RNA was of good quality, since the virus capsid protects the RNA to some extent against degradation by RNases in the early stages of the purification. This single-stranded RNA was used for reverse transcription and the preparation of a cDNA library.

It was also possible to isolate dsRNA from infected leaves using cellulose chromatography. This dsRNA was contaminated to some extent by plant rRNA but was used only in Northern blots and for RACE PCR where plant rRNA contamination posed no problems. Since dsRNA is resistant to degradation by many RNases under conditions encountered during isolation, it was also of good quality. A disadvantage of the dsRNA is evident from Figure 3: whereas
the single stranded RNA bands representing RNA1 and RNA2 are present in approximately equal amounts, there is significantly more RNA3 and RNA4 (Figure 3A). The reason for this is that the coat protein (on RNA4) and movement protein (on RNA3) are required in higher quantities than the replicase components on RNA1 and RNA2. In contrast, in the dsRNA preparations RNA1 and RNA2 are not visible when stained with ethidium bromide, whereas RNA3 is a clear band (Figure 3b).

4.2.0 Monoclonal antibody production

A single hybridoma secreting antibody was obtained from the fusion. This monoclonal antibody (PDA-3C) does not cross react with PNRSV but recognizes all strains of PDV tested. PDV is serologically conserved and it was not anticipated that serological variants would be found. PDA-3C was shown to recognize the coat protein of PDV by an immunoblot.

The hybridoma line producing PDA-3C is stable, divides readily in tissue culture and adequate amounts of antibody could be isolated directly from spent TCS obviating the need for ascites fluid production. It is not clear from this study whether the immunization protocol, using cyclophosphamide to manipulate the immune response, was advantageous.
4.3.0 Triple antibody sandwich ELISA

A reliable TAS-ELISA using PDA-3C was developed to detect the presence of PDV in infected sweet cherry leaves. Although the titre of ilarviruses is high in pollen, most of the assays presented here were performed using young, apical leaves. The reason for this is that leaves were available for a longer period, they were available on all trees regardless of age and they generally gave lower background values by TAS-ELISA than flowers.

The results of the TAS-ELISA performed on the test trees were compared to the results of an independent RT-PCR assay and to a bioassay on 'Shirofugen'. The RT-PCR results were taken to be more reliable than the bioassay results. Although the bioassay is sensitive to all known strains of PDV, the gumming reaction is not always unequivocal. For this reason, the RT-PCR alone was used to identify infected trees. There was a single tree in the sample which was deemed infected by RT-PCR but failed to yield a positive result with the bioassay (see Table 4). A possible reason for this discrepancy is that the titre of PDV may have been too low to elicit a gumming reaction in the 'Shirofugen' assay but was nevertheless high enough to be detected by the more sensitive RT-PCR assay.

A common concern of the use of monoclonal antibody-based testing for plant viruses is the possibility of obtaining false negatives when a serologically distinct strain is
encountered. Strains of PDV appear to be serologically conserved. This is evident from this work where out of 31 trees positive for PDV by RT-PCR, all but one were detected by TAS-ELISA, and from similar findings by other groups using monoclonal antibodies (McMorran & Cameron 1983; Torrance & Dolby 1984) who were able to detect by ELISA all strains of PDV tested. Also, two severe strains of PDV obtained from the virus collection in Wenatchee, WA and unavailable in the virus collection of the Summerland Research Centre, were detected by PDA-3C in the TAS-ELISA. PDA-3C did not recognize PNRSV in the TAS-ELISA, and this is the only other ilarvirus known to infect sweet cherry.

The statistical analysis indicates that the distribution of PDV is irregular within the trees used in this study. Although the majority of the infected trees harboured detectable levels of PDV in at least two-thirds of their leaves, the proportion varied. This is contrary to the uniform distribution of virus reported by Torrance & Dolby (1984). This incongruity may be a reflection of the number of growing seasons since the initial virus infection. The consequences of an uneven virus distribution on the TAS-ELISA results can be partially alleviated by selecting sample leaves from limbs which display characteristic PDV symptoms. Thus, when indexing a mature orchard with a history of PDV, where older infections are expected, the value of Pr could be raised, and fewer leaves collected. In foundation plantings
of virus-free trees, where recent infections would be more prevalent, one would lower the value of Pr and test a greater number of leaves to detect PDV more reliably. Since leaf samples from the same tree can be pooled for TAS-ELISA (Torrance & Dolby 1984), and loaded into the same well in the microtitre plate, assaying more leaves does not significantly increase the scale of the assay. The TAS-ELISA could detect PDV with 99% probability in trees with two-thirds of their leaves infected, if four leaves were assayed at random.

4.4.0 Alternate trapping antibodies

To ensure an indefinite supply of antibodies for the ELISA assay, it was necessary to attempt to design an assay which does not rely on the availability of polyclonal antibodies. Alkaline phosphatase was linked directly to PDA-3C and this conjugate was used in a DAS-ELISA format. Using another approach, the rabbit polyclonal antibodies were replaced with chicken IgY as trapping antibodies. Although the IgY antibodies are polyclonal and hence in limited supply, can be produced in very large quantities.

The most promising assay design used F(ab')₂ fragments to trap PDV in a TAS-ELISA. These are the product of a peptic digest of PDA-3C. This assay gave higher background values than the rabbit polyclonal antibodies used in the TAS-ELISA for this study (Table 6).
4.5.0 The partial nucleotide sequence of RNA3

The partial nucleotide sequence obtained from PDV RNA3 clones is in strong agreement with the published sequence with only seven differences at the nucleotide and three at the amino acid level. Some of these differences may have been due to sequencing errors, especially nucleotide insertions and deletions which would have put the clones out of frame with the published sequence. Not all regions of RNA3 were sequenced from overlapping clones.

The sequence was used to obtain primers for RT-PCR. One of the primer pairs was chosen from within ORF3b (the coat protein) since this sequence is also found on RNA4 and is therefore present in a higher copy number which would make RT-PCR with this pair more sensitive. However, both primer pairs were capable of detecting PDV under all tested conditions and the extra copy-number was not an advantage in this study. The partial sequence was not used for phylogenetic analysis after the full length sequence became available.

4.6.0 The RT-PCR assay

The RT-PCR assay, using one of two primer pairs, was found to be more sensitive than either the TAS-ELISA or the bioassay. Both primer pairs were tested against PNRSV and neither amplified DNA from RNA template from this virus.

Tissue preparation for RT-PCR was more complex because
it was more prone to cross-contamination. This was especially problematic when assaying flower tissue. It is possible that the high titre of virus within the pollen raised the chances of cross-contamination. Assaying unopened buds in winter was also possible but required even more extensive tissue preparation and was not optimal for assaying large numbers of samples. Again, leaves were used for most purposes because of availability and ease of preparation.

When high sensitivity was required, or when results of alternate assays were ambiguous, this was the assay of choice and RT-PCR results were taken to be more accurate than either bioassay or TAS-ELISA results. As with serological assays, there is a concern with PCR based assays that certain minor changes, even point mutations, in the target might lead to false negative results, if they cause the primer to fail to anneal. Using two primer pairs reduces this risk and false negatives from RT-PCR were not encountered in this study.

4.7.0 The complete nucleotide sequence of RNA1

The complete nucleotide sequence of RNA1 was obtained by sequencing eight overlapping clones and 12 RACE PCR clones to obtain the end sequences.

The sequence of RNA1 is in good agreement with other published Bromoviridae RNA1 sequences and the genome organization appears to be the same: RNA1 is monocistronic and the gene product shows extensive sequence homology at the
amino acid level to RNA1 products of other Bromoviridae (viral replicase proteins). The AUGC motifs in the 3'-UTR are also hallmarks of ilarvirus sequences, although these motifs are also found in AMV. The relative positions of the AUGC motifs are similar, and the 3'-end of PDV RNA1 can form essentially the same structure as the 3' end of AMV RNA1 (Figure 11b). In AMV these structures are involved in coat protein binding during genome replication and contain at least two binding sites for the coat protein. (Reusken et al. 1994). The coat protein of the ilar- and alfamovirus genera is required to initiate replication. The coat protein of TSV is able to initiate replication of AMV RNA in the absence of AMV coat protein (Reusken et al. 1995). This implies that the mechanism of replication initiation in ilarviruses and AMV is similar and that the structural elements in the 3'-UTR of PDV may also play an important role in coat protein recognition and in the initiation of replication. However, there is no experimental evidence available for this.

The bromo- and cucumoviruses do not require coat protein to initiate replication and their 3'-UTRs fold into a tRNA-like structure (Perret et al. 1989). These viruses do not have the AUGC motifs and there is no sequence similarity with the ilarviruses or with AMV in the 3'-UTR.
4.8.0 Phylogenetic relationships among Bromoviridae based on the sequence of RNA1

The closer pairing of PDV with AMV than with CiLRV was unexpected, since the latter is an ilarvirus whereas AMV is the only member of the Alfamovirus group. Arguments for the inclusion of AMV in the ilarvirus group have appeared periodically in the literature, but AMV remains the only member of the alfamovirus group because of differences in particle morphology and mode of transmission.

The pairing of AMV with PDV was reproducible and occurred regardless of whether the RNA or the amino acid sequences were analyzed. This indicates that there is a closer phylogenetic relationship between PDV RNA1 and AMV RNA1 than between the RNA1 sequences of PDV and CiLRV. This relationship was not observed using sequence data from RNA3 (Guo et al. 1995). The same phylogeny as in Figure 12A occurs using parsimony analyses of bootstrap replicates made with RNA3 and ORF3a (the putative movement protein; Figure 12B) but not with ORF3b (the coat protein; results not shown). Analysis with ORF3b sequences alone yielded a slightly different consensus tree, but with the fork numbers <50 at some branch points. Grieco et al. (1995) have done a more comprehensive analysis on the phylogenetic relationships of Bromoviridae coat proteins but their analysis did not include PDV.

If viral replicase protein(s) are more reliable than
coat protein(s) for determining phylogenetic relationships among plant viruses, then according to the results presented here, PDV is more closely related to AMV than to CiLRV. It should be noted that RNA2 of Bromoviridae also encodes a protein with a replicase function (Cornelissen et al. 1983b, Ge & Scott 1994) and contains a GDD motif which is highly conserved amongst all RNA viruses (Kamer & Argos 1984). The sequence of PDV RNA2, which is currently unavailable, will help to clarify the phylogeny.

4.9.0 Production and replication of the artificial DI RNAs

It was not possible to synthesize the snapback-type DI RNA as set out in section 2.4.2 and 2.4.3. The PCR primer designed to incorporate the T7 promoter into the template for transcription was highly prone to form secondary structures by self-annealing. Since there was no choice in the sequence of this primer, the only alternative was to optimize the PCR conditions. Even under optimal conditions, the yield of the required bands was low and there were contaminating bands which could not be eliminated. The subsequent ligation of the two PCR products into pUC18, to form a template for the transcription of a snapback-type DI RNA was not feasible. The reasons for this are not clear, but it is possible to speculate that the palindromic DNA sequence in the plasmid was unstable and could not be maintained.

This ligation also failed when it was attempted with
oligonucleotide cassettes attached to pUC18 (sections 2.4.3 and 2.4.4). Since the ligations were very similar it is likely that they failed for the same reason.

Preparation of a deletion-type DI RNA proved to be much simpler, but the DI RNA failed to replicate in vivo in a herbaceous model system. The reasons for this are not known. The Northern blot (Figure 13b) shows the absence of a band in the 1 kb region and shows that levels of viral genomic RNA1 were not affected by the presence of the DI transcript.

The ability of viral RNA to replicate in vivo is sequence dependent. The sequence at the 5’-end is especially important for infectious viral transcripts (reviewed by Boyer & Haenni 1994) and the presence of any non-viral nucleotides usually reduces infectivity. The oligonucleotide cassette at the 5’-end of the insert in pPDVdil was designed to bring the T7 promoter directly adjacent to the first 5’ nucleotide of PDV RNA1 so that there would be no nonviral nucleotides at the 5’-end. The sequence of the template (pPDVdil) was confirmed before transcription to ensure that the ends were unchanged during the plasmid construction.

The transcription of an RNA molecule of the correct size by T7 RNA polymerase was confirmed by denaturing agarose gel electrophoresis. The presence of replicating PDV genomic RNA in the plants was confirmed on the autoradiograph by the presence of an RNA1 band which hybridized to the probe. In a similar study with BMV, Marsh et al. (1991) found that the
length of artificial BMV DI RNA did not greatly affect the level of its own replication although they showed that a smaller DI RNA reduced the synthesis of RNA1 and RNA2 more efficiently than a larger DI RNA. It is therefore unlikely that a DI RNA from PDV of a different size (i.e. a deletion clone of pPDVdi1) would replicate in pumpkins.

4.10.0 Concluding remarks

Two reliable assays to detect PDV in infected fruit trees have been developed. One is a TAS-ELISA which is based on a monoclonal antibody and can detect PDV with 99% confidence in trees which have 2/3 or more of their leaves infected. The assay is suitable for indexing large numbers of trees and the monoclonal antibody reacts against all strains of PDV tested. An RT-PCR assay was also developed and this was found to be more sensitive than the TAS-ELISA and could be used for a longer period during the growing season. This assay was more complex than the TAS-ELISA and not as well suited to indexing large numbers of trees.

The complete nucleotide sequence of PDV RNA1 was also determined. This data was used to investigate the phylogenetic relationship of PDV to AMV and CiLRV. It was shown that, based on this sequence, PDV is more closely related to AMV than to CiLRV. The sequence was also used to construct DI RNA. A deletion-type DI particle was made but failed to replicate in vivo using a herbaceous host.
BIBLIOGRAPHY


