

# Adaptation of plum pox virus to a herbaceous host (*Pisum sativum*) following serial passages

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Plum pox virus (PPV) populations from peaches are able to adapt consistently to herbaceous hosts, characterized by a reduction in time to symptom development, increases in inoculation efficiency and increased titres. PPV adaptation was studied by using pea (*Pisum sativum*) as an alternative host. Two isolates of PPV from peaches were inoculated and passaged in peas ten times using either aphid or mechanical inoculation, generating four independent passage lines. Mechanical-transmission efficiency from peach to pea improved from 3 % at passage 1 to 100 % by serial passage 4 on peas. Inoculation using aphid vectors required six to ten serial passages in pea to reach a peak of 50–60 % transmission efficiency. Sequence analyses of all four PPV population lines inoculated sequentially to pea identified a specific mutation occurring consistently in the NIb gene when compared with the same PPV isolates passaged in parallel in peach. The mutation allowed PPV to replicate up to 20 times faster in the new host. Pea-adapted strains of PPV at every passage were also tested for their ability to infect the original host, peach. Regardless of the number of previous passages, all pea-adapted PPV strains consistently infected peach at low levels using aphid inoculation.

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## INTRODUCTION

Plum pox, also known as sharka, is a major concern to worldwide stone-fruit production. In infected orchards, plum pox leads to a markedly decreased yield due to reduced and deformed fruit production on systemically infected trees. As a result, plum pox can severely limit stone-fruit production in areas where it is endemic. This disease is caused by plum pox virus (PPV, belonging to the family *Potyviridae*), which infects a wide variety of *Prunus* species. Since its initial description in eastern Europe in 1915, it has spread throughout Europe, where it now persists in endemic form in stone-fruit orchards. In 1999, this disease was identified in the USA in the state of Pennsylvania, whereupon an eradication effort was enacted to protect the American stone-fruit industry (Kölber, 2001; Levy *et al.*, 2000; Lopez-Moya *et al.*, 2000; Roy & Smith, 1994).

PPV has a relatively large host range for a potyvirus, infecting plant species in many families and nearly all

members of the genus *Prunus* (Németh, 1986). A large number of herbaceous plant species have also been identified as PPV hosts, both experimentally and as PPV-infected plants in the field. In the current outbreak in Pennsylvania, the approach to control PPV has been the eradication of entire orchards once a single PPV-infected tree has been identified, in an attempt to limit PPV spread by effectively removing a large number of potential *Prunus* hosts. In theory, the removal of a large sector of a primary host species could create selection pressures for PPV to shift hosts, raising the concern that PPV may develop an alternative host as a reservoir. However, despite extensive testing of alternative hosts from within the eradication zone, PPV has been found in only one alternative *Prunus* host (*Prunus glandulosa*) and no alternative herbaceous hosts (J. Halbrecht, personal communication). Similar observations were recorded for herbaceous hosts in the PPV outbreak in Ontario, Canada (Stobbs *et al.*, 2005). However, it should be noted that the reported host range for PPV in the literature reflects the combined experimental and natural host ranges of all of the various PPV isolates studied to date, and that the experimental host range of PPV far exceeds the number of species actually identified as naturally infected hosts in nature.

The GenBank/EMBL/DDBJ accession numbers for the complete genome sequences of PPV isolates PENN3 and PENN4 are DQ465242 and DQ465243, respectively.

A supplementary table showing the primer pairs used in this study is available with the online version of this paper.

During initial characterization studies of Pennsylvania isolates of PPV (Damsteegt *et al.*, 2004), it was observed that PPV isolates from peach (*Prunus persica* L. 'Batsch') were not transferred easily into alternative hosts in the greenhouse. Typically, only a very low percentage of the initially inoculated alternative host species became infected. However, once the alternative host was infected, it became easier to passage the virus within the same host, suggesting that adaptation to the new host may be occurring. Genomic changes in the consensus sequence of genes that encode the RNA replication and intra-plant movement proteins have been observed when viral populations infect a new host (Roossinck & Schneider, 2005).

We hypothesize that selection pressure on PPV for survival in the absence of *Prunus* species associated with an eradication programme might favour PPV adaptation to alternative, non-*Prunus* herbaceous host species. The objective of this study was to examine the potential adaptation of PPV populations undergoing a host shift from a woody *Prunus* host (peach) to an alternative herbaceous host (pea). Pennsylvania isolates of PPV, originally collected from infected peach trees and maintained in peach, were subsequently inoculated to pea (*Pisum sativum* L.), a reported experimental host (Németh, 1986). Multiple serial transmissions to pea using both aphid and mechanical inoculations were conducted to allow the PPV population to adapt fully to its new host. The rates of host adaptation, measured by transmission efficiency and time to symptom development, were compared among PPV isolates transmitted serially by either aphid or mechanical inoculation. The biological data, combined with genomic sequence analysis, provide insights into the adaptation process for PPV and potential risks of host shifts in the eradication zone.

## METHODS

**Plants, viruses and aphid vectors.** For this study, Colmo pea (*Pisum sativum* 'Colmo'; 7–10 days old) and young peach (*Prunus persica* 'Lovell' or 'GF305'; four to ten leaves, less than 25 cm and 1–2 months old) seedlings were used for both mechanical and aphid-vector transmissions. The two PPV isolates used in this study were initially recovered from an infected peach tree in Cumberland County, PA, USA (PENN3; GenBank accession no. DQ465242) or from infected peach fruit collected from an infected tree in Franklin County, PA, USA (PENN4, accession no. DQ465243) by aphid transmission and maintained in peach seedlings (*Prunus persica* 'GF305') using aphid transmission. In all aphid-transmission treatments, *Myzus persicae* (Sulzer) was used as the aphid vector. Aphids were reared on caged turnips (*Brassica rapa* L.) in a growth chamber at 23 °C with a 24 h photoperiod.

**Serial transmissions.** All studies were conducted at the biosafety level 3 containment facility at the USDA facility at Fort Detrick, Frederick, MD, USA. For each of the two PPV isolates, two independent serial-transmission lines were initiated in peas: one aphid-transmitted and the other mechanically transmitted. In parallel treatments done as controls, both isolates were transmitted serially in peach using *M. persicae*. For the initial pea inoculations using PPV-infected peach-leaf tissue as the source, 40–50 pea plants were

inoculated by using either a mechanical or aphid-vector technique as described below. Ten serial pea-to-pea or peach-to-peach transmissions were then conducted, using pooled leaves from all infected plants from the previous passage as a source of virus. After each inoculation, the percentage of plants infected and the minimum time to initial symptom development were noted. In addition, infected pea plants from each serial passage were used as virus-acquisition feeding sources to test the ability of aphids to transmit the pea-acquired viruses back into peach seedlings. RT-PCR to confirm successful infection and quantitative real-time RT-PCR to determine viral titres were conducted by using previously described assays (Gildow *et al.*, 2004; Schneider *et al.*, 2004).

Mechanical inoculations were conducted as described by Martinez-Gomez *et al.* (2000). Briefly, a leaf extract of virus from a previous transmission was made by grinding ten leaves for pea or six to eight leaves (pooled from all infected plants of the previous passage) for peach with a mortar and pestle in approximately 25 ml 0.01 M PBS (pH 7). A light dusting of carborundum was applied to the surface of 20 pea plants or 10 peach seedlings. Approximately 500 µl virus extract was rubbed onto the leaves of the new host plants. Following symptom development, the next serial transmissions were conducted.

Aphid-vector transmissions were conducted as described previously (Gildow *et al.*, 2004). Briefly, PPV-infected pea or peach seedlings were placed in the centre of a clear Lucite box cage (39 × 51 × 37 cm) and surrounded by either ten healthy pea plants or ten peach seedlings. Approximately 500 aphids were placed on the infected plant in each cage and allowed to move freely for 5 days to spread the virus from the source plant to the healthy susceptible seedlings. An insecticide was applied to kill the aphids, and the plants were moved into the greenhouse and observed for symptom formation. Upon symptom development, the next serial transmissions were conducted, using infected peach or pea from the previous transmission as source. To measure the effect of host shift on fitness in the original host, back-inoculations from pea to peach were conducted. Because mechanical transmission of PPV to *Prunus* was inefficient and unreliable, all back-inoculations were by aphid transmission.

**Isolation of total RNA.** Leaf samples from plants infected with each original isolate and after each subsequent transmission for ten serial passages were collected 20–30 days post-inoculation (p.i.) and used for RNA extraction. Ten leaf discs (approx. 50 mg) of infected leaf material were placed into a microcentrifuge tube and flash-frozen by placing in liquid nitrogen. The plant material was ground by using a small pestle, and 200 µl NTS [0.1 M NaCl, 0.01 M Tris (pH 8.0), 1 mM EDTA, 1 % SDS (w/v)] and 200 µl phenol/chloroform (1:1) were added. After centrifugation in a microcentrifuge at maximum speed for 5 min, the supernatant was removed and re-extracted with phenol/chloroform. Sodium acetate solution (25 µl, 0.5 M) was then added with 600 µl 100 % ethanol, and the mixture was placed at –20 °C for 2 h. The solution was centrifuged at maximum speed for 10 min, the supernatant was removed and the pellet was washed with 200 µl 70 % ethanol. The supernatant was poured off and the pellet was dried by vacuum and resuspended in 50 µl distilled H<sub>2</sub>O.

**RT-PCR.** Reverse transcription reactions were conducted following the manufacturer's protocol using SuperScript II reverse transcriptase (Invitrogen), using primers D7, D13, NIBR, and NTR-R (see Supplementary Table S1, available in JGV Online) and 2 µl total RNA template. Ten PCRs were performed by using primer pairs (Supplementary Table S1) designed to cover the entire PPV genome, following the manufacturer's protocol using Platinum PCR Supermix (Invitrogen). Thermal-cycling reactions were conducted for 30 cycles (94 °C denaturation for 30 s, 50 °C annealing for 1 min and 72 °C extension for 1 min). Reactions were checked by using 1 % agarose gel electrophoresis. PCR products were purified by using a MinElute

PCR Purification kit (Qiagen) according to the manufacturer's protocol.

**Sequencing and analysis.** RT-PCR products were generated for passages 5 and 6 for all four pea serial-transmission lines, as well as the fourth serial passage on peach. The purified products were sequenced directly at the USDA Sequencing Facility in Wyndmoor, PA, USA. The consensus sequences of all four pea passages were aligned with the consensus sequence for the serially passaged peach isolates by using CLUSTAL\_W of the sequence analysis software Seqweb/GCG (version 10.0). Differences between the source isolates could be identified easily by comparing the two passaged lines that had been maintained in peach. Mutations that occurred in all four pea-passaged lines, but not in the peach-passaged lines, were identified as being associated with adaptation to pea. These mutation effects were confirmed by checking for reversion of the mutation to the original sequence following back assay, where pea-passaged populations were back-inoculated to peach.

**Protoplast experiments.** Protoplast experiments were conducted to determine whether the new pea-specific mutations were advantageous. PPV virions were isolated from peach and tenth-passage peas for use as source material as described by Laín *et al.* (1988). Pea protoplasts were prepared and inoculated as described previously (Demler *et al.*, 1993; Samac *et al.*, 1983). The viral titre in pea protoplasts 24 h after inoculation was quantified by using real-time RT-PCR as described previously (Schneider *et al.*, 2004)

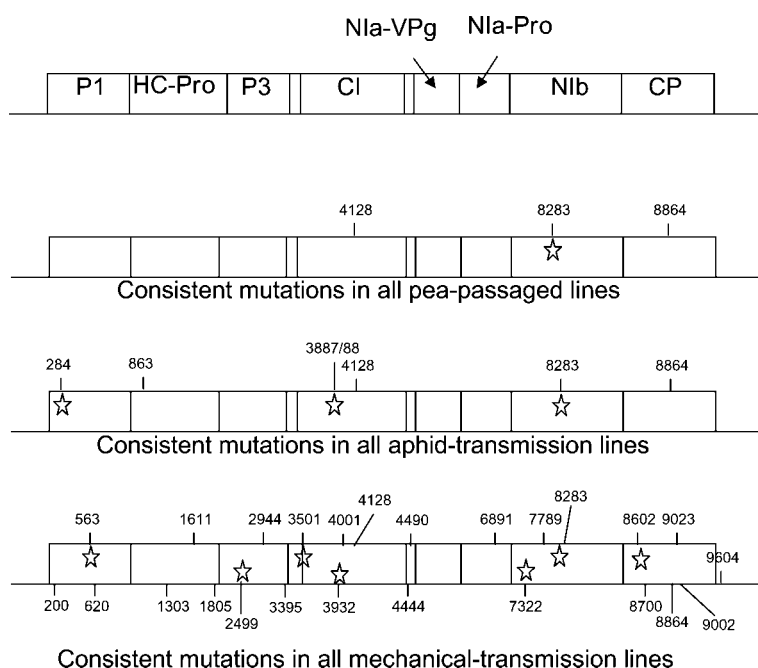
## RESULTS

### Passage on peas

To systematically investigate potential changes in the disease development of PPV in alternative hosts, Colmo peas were inoculated with two peach-maintained PPV isolates. Each isolate was used as an inoculum for two pea lines, one aphid-inoculated by using *M. persicae* and the

other mechanically inoculated (Fig. 1). The infected peas were then used as source for a series of nine additional passages, with the infected pea hosts from the previous passage used as an inoculum source for the next passage. The percentage of successfully inoculated pea plants was recorded after each transmission (Table 1). Following the first passage from peach to pea hosts, only 3–5 % of inoculated peas became infected in both the aphid- and mechanical-inoculation treatments. Aphids transmitted PPV successfully to 50–80 % of peach seedlings, using the same PPV-infected peach source material in parallel treatments as positive controls. These numbers were consistent between the two isolates and within passage lines of the same isolate. After the initial passage from peach to pea (passage 1), the number of pea plants infected after each successive passage from pea to pea increased. Mechanical transmission of PPV resulted in more rapid adaptation to the new pea hosts than aphid transmission. When mechanical inoculation was used to inoculate plants, the number of pea plants successively infected increased to 75 % by passage 3, whereas only 15–20 % of pea plants were infected when aphid vectors were used to inoculate the peas. After the fourth passage, mechanical inoculation always resulted in 100 % successful transfer of PPV. However, the aphid-inoculation success rate remained consistently lower and never exceeded 60 %, up to and including passage 10. The results were very similar for both PPV isolates used.

Time to initial symptom development p.i. was also compared in the pea-passaged lines (Table 2). In initial passages, symptom development was not detected until 30 days p.i. The time required for initial symptom development generally decreased with passage from pea



**Fig. 1.** Locations of mutations accumulated in passage experiments. A schematic diagram of the PPV genome with genes labelled is shown at the top. Maps of the mutations that occurred consistently in all pea-passaged lines, in all aphid-transmitted lines and in all mechanically transmitted lines are shown below. Mutations that resulted in amino acid changes are signified by stars on the genome maps.

**Table 1.** Percentage infection when inoculating peas with PPV from peaches

PPV isolate	Passage					
	1	2	3	4	5	10
<b>Aphid-transmitted</b>						
PENN3	1/40 (2.5 %)	4/20 (20 %)	3/20 (15 %)	2/10 (20 %)	3/10 (30 %)	5/10 (50 %)
PENN4	2/40 (5 %)	2/20 (10 %)	2/10 (20 %)	3/10 (30 %)	5/10 (50 %)	6/10 (60 %)
<b>Mechanically transmitted</b>						
PENN3	2/50 (4 %)	4/20 (20 %)	7/10 (70 %)	9/10 (90 %)	10/10 (100 %)	10/10 (100 %)
PENN4	1/50 (2 %)	4/20 (20 %)	8/10 (80 %)	9/10 (90 %)	10/10 (100 %)	10/10 (100 %)

to pea until it reached a minimum of 11 days by passage 10. Unlike transmission efficiency, the aphid-vectored and mechanically inoculated PPV populations had similar decreases in their times to symptom development, suggesting that time to symptom development may be driven by selection pressures independent of vectoring. Real-time RT-PCR assessments of PPV titres 30 days p.i. indicated that PPV accumulated to lower levels in the initially inoculated peas, but titres generally increased with continued passage on peas (Table 3). The cycle threshold ( $C_t$ ) values had a downward trend and were significantly lower at the end of the passing regime for all four lines, indicating increased viral titres and suggesting improved replication and/or movement. The largest difference in  $C_t$  values occurred consistently between the first and second passage. A similar pattern was not seen in the peach-passaged line, as  $C_t$  values indicating viral titres remained relatively unchanged (data not shown). Once again, similar trends were seen in both PPV isolates used.

### Peach back-inoculations

PPV-infected pea seedlings from each passage were used as virus-acquisition sources to test the ability of aphids to transmit (back-inoculate) the pea-adapted PPV back into peach hosts (Table 4). A general decrease in the success of back-inoculation was observed as PPV viral populations were passaged on peas, from 90 % success in the initially inoculated pea populations to 30–50 % by passage 8 for the aphid-vectored PPV lines. The mechanically inoculated

lines had an even more dramatic reduction in the ability to reinfect peach than the aphid-vectored lines, dropping from 70 % at passage 1 to 10–15 % after passage 8. The pea-passaged populations never lost the ability to infect peach and continued to be infectious to peach at low levels (10–20 %), even after as many as 40 passages on pea (data not shown).

### Sequence analysis

The consensus sequences (representing the most common sequence in a given viral population) of the pea-passaged populations were compared with the original PPV source sequences (either PPV-PENN3 or PPV-PENN4), originating and maintained in peach, to determine what mutations had occurred in response to host shifting. The consensus sequences were obtained by direct sequencing of RT-PCR products, using extracted total RNA from infected leaves as template. As a control, the peach-passaged populations were sequenced after four passages. For both the aphid-inoculated and the mechanically inoculated pea-passaged lines, extractions were taken from passages 5 and 6 (deposited in GenBank under accession numbers EF611241–EF611248). These passages were chosen for sequencing because they represented points in the passing experiments where major phenotypic improvements had already occurred, suggesting that the populations were adapted to the new host. Two consecutive passages were sequenced to confirm that mutations were fixed and remained stable in the populations.

**Table 2.** Time (days) to symptom development for isolates PENN3 and PENN4 during passing on pea

PPV isolate	Passage					
	1	2	3	4	5	10
<b>Aphid-transmitted</b>						
PENN3	31	22	16	14	14	14
PENN4	30	20	14	15	13	14
<b>Mechanically transmitted</b>						
PENN3	28	16	14	14	14	11
PENN4	28	21	17	15	16	12

**Table 3.** PPV quantification ( $C_t$  value) in pea-passaged line plants 30 days p.i., using real-time RT-PCR

PPV isolate	Passage					
	1	2	3	4	5	10
<b>Aphid-transmitted</b>						
PENN3	33.12	21.16	16.67	19.83	18.85	16.53
PENN4	30.82	24.94	22.20	19.98	20.37	17.81
<b>Mechanically transmitted</b>						
PENN3	28.33	20.91	25.82	17.21	16.34	13.55
PENN4	32.49	22.32	22.01	21.19	19.78	15.26

**Table 4.** Percentage infection when back-inoculating peaches with PPV from individual pea passages

PPV isolate	Passage					
	1	2	3	4	5	8
<b>Aphid-transmitted</b>						
PENN3	9/10 (90 %)	13/20 (65 %)	10/20 (50 %)	10/20 (50 %)	8/20 (40 %)	10/20 (50 %)
PENN4	10/10 (100 %)	7/10 (70 %)	6/10 (60 %)	3/10 (30 %)	9/20 (45 %)	7/20 (35 %)
<b>Mechanically transmitted</b>						
PENN3	7/10 (70 %)	4/10 (40 %)	7/20 (35 %)	6/20 (30 %)	2/10 (20 %)	1/10 (10 %)
PENN4	7/10 (70 %)	2/10 (20 %)	6/10 (60 %)	3/10 (30 %)	3/10 (30 %)	3/20 (15 %)

When compared with the original PPV isolates maintained in peach, numerous fixed mutations were observed in the individual pea-adapted lines, particularly the mechanically transmitted lines. The mechanically transmitted pea-passaged PPV-PENN3 line had the most mutational differences, with 159 nucleotide changes (compared with the PPV-PENN3 sequence from peach), resulting in 32 amino acid changes scattered through the genome. However, only a limited number (22) of these changes also occurred in the mechanically transmitted pea-passaged PPV-PENN4 line (compared with the PPV-PENN4 sequence from peach).

Three nucleotide changes and one amino acid change were common to both the mechanically and aphid-transmitted pea-passaged PPV populations (Fig. 1), with the amino acid change occurring in NIb (nt 8283, amino acid N→I). Silent mutations occurred in the CI coding region (nt 4128) and in the NIb coding region (nt 8602). Four nucleotide changes, leading to two amino acid changes, occurred consistently in all four aphid-vectored PPV populations (Fig. 1). The amino acid changes unique to aphid transmission were in the P1 (nt 284, amino acid K→R) and CI (nt 3887 and 3888, amino acid H→S) coding regions of the genome, and the silent mutation occurred in the HC-Pro gene (nt 863). There were 22 mutations that only occurred in both of the mechanically transmitted pea-passaged lines (Fig. 1), 16 of which were silent. Interestingly, one amino acid substitution occurred immediately prior to the aphid transmission-associated DAG motif (Lopez-Moya *et al.*, 1999) in the CP protein region (nt 8864, amino acid V→I). No non-synonymous mutations were noted in the HC-Pro coding region, which is also required for aphid transmission.

### Replication in pea protoplasts

In order to determine whether the mutation in the NIb protein could be associated with more efficient replication in peas and host adaptation, PPV populations from pea-passaged and peach-passaged lines were inoculated to pea protoplasts. Virions were isolated from the tenth passage of PPV-PENN4 on peas and from the original PPV-PENN4 source peaches. The populations were sequenced to confirm the presence of the NIb mutation in the pea lines

and the lack of the NIb mutation in the peach lines. The virion titres were analysed by using a spectrophotometer, and equal titres of virions (0.1 µg) were used to inoculate pea protoplasts with or without CaCl<sub>2</sub>. In both mock-inoculated (no CaCl<sub>2</sub>) protoplast samples, no virus replication was detected by real-time RT-PCR 24 h after infection (Table 5). In the pea-protoplast sample inoculated by peach-derived virions, the titre of PPV was much lower ( $C_t$  29.8) than in the protoplast sample inoculated with pea virions ( $C_t$  20.1), equivalent to an approximately 20-fold increase in replication compared with a standard curve (data not shown).

## DISCUSSION

Plant viral adaptation to hosts has been reported often in the literature (reviewed by Garcia-Arenal *et al.*, 2001; Roossinck & Schneider, 2005) and is an obvious factor in the success of these pathogens. The issue is of particular importance in the case of PPV in the USA, where eradication efforts continue. This concept is significant, because the success of the eradication effort is contingent on PPV remaining in *Prunus* hosts. To date, PPV has been identified in only one non-commercial stone fruit in Pennsylvania (J. Halbrecht, personal communication).

In preliminary experimental host-range work (V. D. Damsteegt, unpublished data), Pennsylvania isolates of PPV demonstrated consistent improvement when passaged

**Table 5.** Real-time RT-PCR quantification of PPV accumulation in pea protoplasts

Inoculum*	$C_t$ value
None	No product
PPV pea virions mock	No product
PPV peach virions mock	No product
PPV pea virions	20.11
PPV peach virions	29.82

\*PPV pea virions were purified from passage 10 pea populations; PPV peach virions were purified from passage 4 peach populations; mock inoculations did not include CaCl<sub>2</sub>.

on alternative herbaceous hosts, especially peas. In the initially inoculated plants, the percentage of successful infection was quite low, but with continued passage on the same host, inoculation success increased. In many herbaceous hosts, continued passage of PPV also resulted in increased symptom severity and decreased time to the appearance of symptoms. The sources of the isolates used for these host-range experiments were peach tissue taken directly from the quarantine zone in Pennsylvania. This peach tissue was always grafted onto healthy peach 'GF305' for use in further experiments. The natural history of these isolates is unknown. However, the consistent pattern of improvement associated with passaging in a new host suggested the possibility that the Pennsylvania PPV isolates were adapting to their new hosts.

The pattern of 'improvement' held up consistently when peas were analysed for these traits using four independent PPV passage lines. In the initial transfer from peach to pea, PPV had very low infection rates (3–5%) and caused very mild symptoms that did not appear until 30 days p.i. As PPV populations were passaged from pea to pea, the infection rates increased and the time to symptom development became shorter (Tables 2 and 3). As time to symptom development decreased, symptom severity increased on the new pea host (data not shown). In addition, viral titres increased with continued passaging on peas (Table 3). All of these parameters indicated strongly that PPV populations were adapting to the new host.

Dramatic differences were not observed either in the time to symptom development or in titre development once peas were infected by either aphid transmission or mechanical inoculation. In contrast, there were distinct differences in transmission efficiency between the PPV lines that were aphid-transmitted and those that were mechanically transmitted. The mechanically transmitted lines showed more dramatic improvements in inoculation success than the aphid-transmitted lines (Table 1). This suggests strongly that PPV also underwent significant adaptations specific to the mode of transmission. It can be assumed that the PPV isolates used were primarily aphid-vectored or graft-transmitted in the field, based on the fact that successful mechanical inoculation of *Prunus* is extremely difficult. It is quite possible that most of the dramatic improvement seen in inoculation success (Table 1) in mechanically transmitted PPV lines is actually adaptation to mode of transmission, not host adaptation. However, there may also be bottleneck effects associated with aphid transmission that would limit the rate of adaptation. The method of mechanical inoculation ensures that more virus is transferred to the healthy seedlings by mechanical inoculation than by aphid inoculation. It is impossible to estimate the percentage of transferred virus that is able to initiate infection for either inoculation method without additional experiments.

Sequence analyses of the four pea-passaged PPV populations revealed the presence of three consistently occurring

mutations. One of these changes resulted in an amino acid change in the NIb protein. A change in the NIb coding sequence indicates that positive selection may have occurred to allow for better interaction with the new host, as the NIb protein is the RNA-dependent RNA polymerase that is involved directly in replication of the viral genome with the assistance of various host components (Shukla *et al.*, 1994). The silent mutations in the CI and NIb coding regions are harder to suggest a mechanism for, although the repeated occurrence suggests selection pressure for the mutations to occur. The CI protein is involved in cell-to-cell movement (Carrington *et al.*, 1998) and replication (Fernandez *et al.*, 1997), but that would not explain the value of a silent mutation. Both aphid- and mechanically transmitted PPV pea-passaged lines demonstrated the greatest level of improvement in the second passage, suggesting that the mutations that made the greatest difference in fitness in pea had occurred and become prevalent in the initially inoculated (passage 1) plants. Subsequent sequence analysis of the NIb region of the initially inoculated plants did confirm the presence of the mutation in all four lines (data not shown).

The improved pea-infecting phenotype demonstrated by PPV upon passaging in peas is probably due to an increase in the percentage of individuals in the viral population that are more compatible with the new pea host components required for replication and intercellular movement. If a virus population can adapt to replicate more efficiently in a new host, then it may be more successful at cell-to-cell and long-distance movement, as well as plant-to-plant transmission. These factors would select for a higher probability of virus survival. Protoplast assays indicate that PPV populations passaged in peas are much better at replicating in peas than PPV populations from peaches (Table 5). In addition, a virus population more efficient at cell-to-cell and systemic movement would result in faster dissemination throughout the plant and symptom development in a shorter time, as seen in pea-adapted PPV (Table 2).

Interestingly, there appears to be a limited trade-off effect on PPV fitness in peach associated with adaptation to peas. PPV passaged on peas by aphids seems to lose little of its effectiveness in infecting peaches. The initial back-inoculation to peach (Table 4) from the first passage in pea was very successful (90%). Although there was a decrease in transmissions back to peach by the second passage, the aphid-transmitted lines stabilized at about 50% transmission in their ability to reinfect peach. This is significant, because PPV spread in nature from putative herbaceous alternative hosts back to peach would probably be limited to aphid transmission. In the mechanically inoculated lines, there was a far greater drop-off in success of back-inoculation, from 10 to 20% by passages 7 and 8, probably due to adaptation to mechanical transmission. However, PPV passaged serially on peas never lost the ability to infect peach, even after 40 passages.

Several mutations were conserved between the two mechanically transmitted pea populations, including a

mutation near the DAG motif. It has been determined that this DAG motif is critical to aphid transmission of potyviruses (Pirone & Perry, 2002) and that it also plays a role in cell-to-cell movement (Andrejeva *et al.*, 1999). However, PPV that was passaged mechanically up to 40 times never lost the ability to be aphid-transmitted, suggesting that this mutation does not actually abolish aphid transmission. Because these isolates were sequenced at the PCR product level, only the consensus sequence was determined, so it remains possible that a smaller subset of the mechanically transmitted PPV populations retained the original sequence at this site. There were also mutations that occurred in all aphid-transmitted lines, suggesting adaptation to our vector of choice, *M. persicae*. Theoretically, the two modes of transmission should be independent and selection for mechanical transmission would not affect transmission by aphids. It is interesting to note that mechanically transmitted lines accumulated far more fixed mutations. The fact that these mutations did not occur consistently in both mechanically passaged isolates suggests that most of these mutations are the effect of drift. Why this would be the case for mechanical transmission and not for aphid transmission remains to be determined.

In conclusion, a specific mutation associated with adaptation to peas has been identified for PPV isolates from Pennsylvania. The fact that PPV seems to undergo similar improvement in infection rates, titres and symptom development in all herbaceous hosts examined so far suggests that the virus must adapt to each new host. Inefficient transmission to and replication in a new host may explain the failure to detect PPV in native plant and herbaceous host sentinel plant surveys conducted in the quarantine zone in Pennsylvania.

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