Evidence for heterologous encapsidation of potato spindle tuber viroid in particles of potato leafroll virus

Maddalena Querci,1 Robert A. Owens,2 Ida Bartolini,1 Vidal Lazarte1 and Luis F. Salazar1

1 Molecular Virology Laboratory, International Potato Center, PO Box 1558, Lima, Peru
2 Molecular Plant Pathology Laboratory, Beltsville Agricultural Research Center, Beltsville, MD 20705, USA

The aphid Myzus persicae (Sulz.) was shown to transmit potato spindle tuber viroid (PSTVd) to potato clone DTO-33 from source plants doubly infected with potato leafroll virus (PLRV) and PSTVd. Transmission was of the persistent type and did not occur when the insects were allowed to feed on singly infected plants. Only low levels of PSTVd were associated with purified PLRV virions, but its resistance to digestion with micrococcal nuclease indicates that the viroid RNA is encapsidated within the PLRV particles. Epidemiological surveys carried out at three locations in China revealed a strong correlation between PSTVd infection and the presence of PLRV, suggesting that PLRV can facilitate PSTVd spread under field conditions.

Like all known viroids, potato spindle tuber viroid (PSTVd) is an independently replicating agent which completes its infection cycle without generating either a capsid or other viroid-specific proteins. Its genome is a small (359 nt), single-stranded, covalently closed circular RNA molecule whose extensive regions of intramolecular complementarity are responsible for its unusual stability in vivo (Sänger et al., 1976; Riesner, 1987).

In potato, natural spread of PSTVd has been repeatedly shown to occur either by foliar contact (Goss, 1926; Merriam & Bonde, 1954) or botanical seed (Hunter et al., 1969). Early reports (Goss, 1930) suggested that PSTVd might be transmitted by chewing-insects, but these reports have not been confirmed. De Bokx & Piron (1981) reported a low rate of transmission by the aphid Macrosiphum euphorbiae (Thomas) but no transmission was obtained with either Myzus persicae (Sulz.) or Aulacorthum solani (Kaltenbach).

In contrast to PSTVd, potato leafroll virus (PLRV); a member of the genus Luteovirus is readily aphid transmitted, M. persicae being the most efficient natural vector (Goss, 1930). Like all luteoviruses, PLRV is restricted to the phloem tissue of infected plants (Casper, 1988), and aphid transmission is both persistent and nonpropagative (Eskandari et al., 1979). PLRV has a narrow host range, and the virus is commonly found wherever potatoes are grown.

Salazar et al. (1995) have recently reported that M. persicae could transmit PSTVd to potato and other test plants only when the source plant was doubly infected with PLRV and PSTVd. In some experiments, the percentage of PSTVd transmission reached 100%; no transmission was observed from source plants infected with the viroid alone, however. Here, we present results of experiments designed to elucidate the mechanism responsible for aphid transmission of PSTVd and discuss the possible epidemiological implications of our findings. A preliminary report of this work has been published elsewhere (Querci et al., 1996).

As described earlier (Salazar et al., 1995), occasional PSTVd contamination of PLRV isolates maintained at the International Potato Center (CIP), Lima, Peru provided the first indication that PLRV might facilitate aphid transmission of PSTVd. Additional epidemiological evidence for an association between PSTVd and PLRV in field-grown potatoes was subsequently obtained by testing plants growing at several locations in China for the presence of PSTVd and/or PLRV. A total of 880 plants from three locations was tested by nucleic acid spot hybridization (NASH) in 1994, and the results from these analyses are summarized in Table 1.

Rates of infection for both PLRV and PSTVd were quite variable, ranging between 3.6–86% and 0.9–27.2%, respectively. Vector pressure was highest at the Bashang Institute (Hebei province), and as expected, the incidence of PLRV at that location appeared to be somewhat greater than at the other two locations. Most (but not all) PSTVd-infected plants at all three locations were also infected with PLRV. Exceptions included small numbers of PSTVd-infected Bashu No. 10 plants growing at the Bashang Institute and Purple flower white plants growing in Wachuan county. Because the incidence of PSTVd for these two genotype–location combinations was quite low (0.9–1.7%), such a result was not unexpected. No effect on either host genotype or location was identified for the mechanism responsible for aphid transmission of either PLRV or PSTVd, and chi square ($\chi^2$) analysis of the pooled data was performed to test the independence of PSTVd and PLRV distribution. The $\chi^2$ value
Table 1. Incidence of PLRV and PSTVd in three potato-growing locations in China

Field surveys were done in 1994. Samples from the Bashang Institute and Wachuan county were obtained from farmers’ fields. All samples were randomly selected, and the presence or absence of PLRV and/or PSTVd was determined by NASH. Numbers of singly and doubly infected plants are shown in parentheses. Analysis of all data (including a Yate’s correction) yielded a \( \chi^2 \) value of 29.68. Assuming no effect of host variety or location on aphid transmission, a \( \chi^2 \) value of 6.635 indicates significance at the 1% level (Steele & Torrie, 1960).

<table>
<thead>
<tr>
<th>Potato variety</th>
<th>Location</th>
<th>No. of samples</th>
<th>Infection rate (PLRV)</th>
<th>Infection rate (PSTVd)</th>
<th>Coinfection rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tiger head</td>
<td>Bashang Inst., Hebei province</td>
<td>132</td>
<td>66.7% (88)</td>
<td>2.3% (3)</td>
<td>2.3% (3)</td>
</tr>
<tr>
<td>88-1-19</td>
<td>Bashang Inst., Hebei province</td>
<td>114</td>
<td>86.0% (98)</td>
<td>2.6% (3)</td>
<td>1.8% (2)</td>
</tr>
<tr>
<td>Bashu No. 10</td>
<td>Bashang Inst., Hebei province</td>
<td>106</td>
<td>63.2% (67)</td>
<td>0.9% (1)</td>
<td>0% (0)</td>
</tr>
<tr>
<td>Purple flower white</td>
<td>Wachuan county, Inner Mongolia</td>
<td>241</td>
<td>17.8% (43)</td>
<td>1.7% (4)</td>
<td>0% (0)</td>
</tr>
<tr>
<td>Desiree</td>
<td>Wachuan county, Inner Mongolia</td>
<td>56</td>
<td>3.6% (2)</td>
<td>0% (0)</td>
<td>0% (0)</td>
</tr>
<tr>
<td>Tiger head</td>
<td>Inner Mongolia University</td>
<td>103</td>
<td>68.9% (71)</td>
<td>27.2% (28)</td>
<td>25.2% (26)</td>
</tr>
<tr>
<td>Purple flower white</td>
<td>Inner Mongolia University</td>
<td>128</td>
<td>36.7% (47)</td>
<td>18.8% (24)</td>
<td>18.8% (24)</td>
</tr>
</tbody>
</table>

Table 2. Aphid transmission of PLRV and PSTVd to potato variety DTO-33

In rows 3 and 4 of the Table, aphids were allowed two acquisition feedings, i.e. the first on PLRV-infected and the second on PSTVd-infected source plants.

<table>
<thead>
<tr>
<th>Inoculum source(s) Passage</th>
<th>Successful transmission</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PLRV</td>
</tr>
<tr>
<td>PLRV</td>
<td>No</td>
</tr>
<tr>
<td>PLRV</td>
<td>Yes (2/5)</td>
</tr>
<tr>
<td>PLRV → PSTVd</td>
<td>Yes (2/3)</td>
</tr>
<tr>
<td>PLRV → PSTVd</td>
<td>Yes (1/5)</td>
</tr>
<tr>
<td>PLRV + PSTVd</td>
<td>Yes (4/5)</td>
</tr>
<tr>
<td>PLRV + PSTVd</td>
<td>Yes (1/5)</td>
</tr>
</tbody>
</table>

* Data presented as no. infected plants/no. inoculated plants.

(29.68) was highly significant, strongly suggesting that PLRV facilitates the spread of PSTVd under field conditions.

Additional aphid transmission experiments were carried out to more precisely characterize the mode of PSTVd transmission by *M. persicae*. Potato clone DTO-33 (CIP No. 800174), a *Solanum tuberosum × S. andigena* hybrid susceptible to PLRV and PSTVd, was used as both inoculum source and test plant. Plants doubly infected with PLRV and PSTVd were obtained by initial inoculation with PLRV by means of its vector, *M. persicae*, and subsequent manual inoculation with sap from PSTVd-infected tomato (*Lycopersicon esculentum* cv. Rutgers). PLRV isolate 01 was provided by Upali Jayasinghe (CIP), whereas a ‘severe’ strain of PSTVd was isolated at CIP in 1983. After inoculation, plants were maintained in a growth chamber at 18–22°C with constant relative humidity (80%) and a 12 h photoperiod (light intensity = 27 µE/m²/s) and periodically tested for PSTVd and PLRV infection as indicated below.

Aphid colonies from a non-viruliferous colony of *M. persicae* were raised on Chinese cabbage (*Brassica pekinensis* (Lour.) Rupr.) under greenhouse conditions. One month after PLRV or PSTVd inoculation, apherous aphids were allowed a 3 day acquisition access period on either singly or doubly infected source plants. Aphids (five aphids per plant) were then transferred to young uninfected potato plants by placing the insects on filter paper squares using a sterile brush and then allowing them to walk onto a plant leaf. A final 2 day passage on healthy Chinese cabbage was included in one experiment. After a 3 day transmission access period (TAP), aphids were killed by spraying plants with a contact insecticide.

Fifteen and 45 days after the TAP, inoculated plants were tested for the presence of PSTVd and PLRV using a combination of NASH and ELISA (Clark & Adams, 1977). NASH tests for PSTVd and PLRV (Querci *et al.*, 1995) were...
PLRV/PSTVd transencapsidation

M. persicae was able to transmit PLRV under all conditions tested. PSTVd transmission, in contrast, was observed only when the aphids were allowed to acquire the viroid from doubly infected source plants. Comparison of data presented in rows 3 and 4 of the Table shows that aphids allowed to feed sequentially on plants infected with PLRV and PSTVd alone were unable to transmit PSTVd. Feeding on healthy cabbage prior to transfer to the test plants reduced but did not eliminate the ability of aphids to transmit PSTVd from doubly infected source plants. Thus, PLRV-mediated transmission of PSTVd by M. persicae appears to be of the persistent type.

To further characterize the nature of the association between PSTVd and PLRV, a series of virus purifications was carried out using leaf tissue collected from singly and doubly infected potato plants. The first such experiment compared the amounts of PSTVd RNA associated with virions purified from plants infected with both PLRV and PSTVd with those associated with particles isolated from a pooled sample of leaves collected from singly infected plants. PLRV was purified as described by Salazar (1995), and encapsidated RNAs were recovered by phenol–chloroform extraction and ethanol precipitation. RNA pellets were resuspended in sterile nuclease-free water and reverse-transcribed using the GeneAmp RNA PCR kit (Perkin-Elmer) and a random hexanucleotides mixture (pdN6) according to the supplier's protocol. PSTVd-and PLRV-specific cDNAs were then amplified in separate PCR reactions using appropriate pairs of oligonucleotide primers: RAO-2 (5′ GCGGATCCGGTGGAAACAACTGAAGC 3′) and RAO-33 (5′ GCCGGTACCAGTTCGCTCCAGGTTTCCCC 3′) for PSTVd (Owens et al., 1990), and LR210 (5′ TAGCATGCCAGTGGTTAGTGTC 3′) and LR211 (5′ GCCCTCGAGTCTACCTATTTGG 3′) for PLRV (Robertson et al., 1991). Amplifications (40 cycles) were carried out using a 94 °C (1 min), 55 °C (2 min), 72 °C (1 min) profile followed by a 5 min final extension at 72 °C, and PCR products were visualized by electrophoresis on 5% acrylamide gels. Predicted sizes for the PSTVd- and PLRV-specific PCR products are 226 and 534 bp, respectively.

As shown in Fig. 1(a), large amounts of a PLRV-specific
product of the appropriate size were present in PCR reactions containing RNA derived from either singly (lane 3) or doubly (lane 2) infected plants. The corresponding PSTVd-specific product was present only in reactions containing RNA derived from doubly infected tissue (lane 5). When virions were isolated from a mixture of leaves collected from singly infected plants, no PSTVd-specific PCR product was produced (compare lanes 5 and 6).

To determine whether transencapsidation of PSTVd by PLRV was responsible for its observed aphid transmissibility, two different types of samples were treated with micrococcal nuclease: (i) PLRV virions isolated from doubly infected leaf tissue and (ii) mixtures of virions isolated from singly infected plants plus sufficient PSTVd RNA to produce a comparable ratio of PSTVd/PLRV (as measured by NASH). Trial experiments showed that 15 min incubation at 30 °C with 1 × 10−13 units/µl micrococcal nuclease completely destroyed concentrations of PSTVd similar to those found in PLRV virions isolated from doubly infected tissue (results not shown).

Parallel incubations were carried out, each containing the same amount of virus but only one containing micrococcal nuclease. After incubation, the undigested RNAs were isolated by phenol–chloroform extraction, reverse transcribed, and analysed by RT–PCR. Results from these analyses are presented in Fig. 1 (b, c).

Each set of five amplifications (lanes 1–5 and 6–10) contained a series of 3-fold dilutions of the initial preparation of randomly primed PLRV and PSTVd cDNAs. Samples in lanes 1–5 were derived from untreated virions, while those in lanes 6–10 were treated with micrococcal nuclease before RNA extraction. In Fig. 1(b), comparison of lanes 1–5 with 6–10 shows that, as expected, the viral genomic RNA was resistant to micrococcal nuclease digestion. The corresponding PSTVd-specific analyses are shown in Fig. 1(c). Because the virions contain relatively low levels of viroid RNA, the amount the PSTVd-specific PCR product could be seen to decrease as the amount of randomly primed cDNA template was reduced. Micrococcal nuclease digestion had no effect on the amount of PSTVd-specific PCR product synthesized, however.

This experiment was repeated several times with different virus preparations, and though the relative amount of PSTVd in the different PLRV preparations was found to vary, the results obtained were consistent and comparable. The simplest explanation for our results is that the PSTVd associated with virions isolated from doubly infected plants is, like the PLRV genomic RNA, located within the virus particle. Unencapsidated PSTVd RNA would have been degraded by the micrococcal nuclease digestion. Expressed on a molar basis, virions from doubly infected plants contained approximately one molecule of PSTVd for every 3000–5000 molecules of PLRV RNA.

In nature, plant viruses and other subviral pathogens (i.e. satellite/defective interfering RNAs or viroids) have many opportunities for interaction (Falk & Bruening, 1994). Multiple virus infections are a common occurrence in crop and weed hosts, and such a situation may result in virus particles in which (i) individual particles contain structural proteins derived from more than one virus or (ii) the genome of one virus is encapsidated in the structural proteins of another. This phenomenon, variously known as transencapsidation, genomic masking or phenotypic mixing, has been observed many times for members of the luteovirus and potyvirus groups (Falk et al., 1995). Several sobemoviruses are known to support the replication of small viroid-like satellite RNAs, and Francki et al. (1986) showed that velvet tobacco mottle virus (VTMoV) is able to encapsidate PSTVd. More recently, Miller et al. (1991) have reported the association of a small, ribozyme-containing satellite RNA with certain isolates of barley yellow dwarf virus-RPV serotype. PSTVd is similar in size to the barley yellow dwarf virus satellite RNA (359 vs 322 nt), and our data show that it can also be encapsidated by a luteovirus, PLRV.

Transencapsidation of PSTVd by an assistior virus and the resulting acquisition of aphid transmissibility have important epidemiological implications. Viroids are generally thought to be mechanically transmitted (Diener, 1987), but as pointed out by Francki et al. (1986), ‘A scenario can be envisaged where a viroid which originated in one plant species in which it was symptomless could be encapsidated by a virus and thence vectored to another species, not necessarily a host of the virus, but one in which the viroid could become pathogenic’. Our survey data from China indicate that, even where the overall level of PLRV infection was relatively moderate, all or almost all plants found infected with PSTVd were also infected with PLRV. VTMoV greatly suppresses PSTVd replication in Nicotiana clevelandii; nevertheless, tomato plants which are immune to VTMoV became infected with PSTVd when inoculated with viroid-containing VTMoV preparations (Francki et al., 1986). Additional data will be required to determine whether PLRV has a similar effect on PSTVd replication in potato, but transmission by aphids or other insect vectors provides a plausible explanation for the presence of PSTVd in such ‘atypical’ hosts as avocado (Querci et al., 1995).

Aphid transmission of PSTVd may be most important in long distance dissemination of the viroid, facilitating its spread and greatly reducing the possibility of its control. More than 70 years ago, Schultz & Folsom (1923) reported the transmission of PSTVd (‘spindling tuber’) in cv. Green Mountain by M. euphorbiae from an inoculum source containing ‘leafrolling mosaic’ (probably PLRV plus a combination of potato viruses X and Y). More recently, De Bokx & Piron (1981) reported low levels of non-persistent transmission of PSTVd by M. euphorbiae but not by M. persicae or A. solani. Our failure to detect transmission of PSTVd by M. persicae from source plants infected with PSTVd alone may reflect transmissibility differences among aphid species.

An important step in breeding for virus resistance is the identification of specific factors which tend to break/overcome that resistance. In the case of PLRV, no genes conferring
immunity have been described, and the various resistance genes known appear to act by different mechanisms (Barker & Harrison, 1985).

Salazar et al. (1995) have reported that the presence of PSTVd can lead to a decrease in the level of resistance to PLRV. Knowledge of how other pathogens such as PSTVd might interfere with the expression of genes conferring PLRV resistance would help in selection of the type of resistance to be incorporated and predicting its expected durability. Efforts to identify the different components of resistance to PLRV as well as to elucidate the role of PSTVd in decreasing such resistance are currently underway.

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References


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