Detection and Quantification of Plum Pox Potyvirus in Aphid Vectors by Real-Time Fluorescent Reverse Transcription-PCR

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Abstract
A real-time, fluorescent, reverse transcription-polymerase chain reaction (RT-PCR) assay was developed for the detection of Plum pox potyvirus (PPV). The methods developed are reproducible, specific to PPV, and sensitive enough to consistently detect PPV transcripts at the 10 fg level. The assay is more sensitive than either ELISA or traditional PCR followed by electrophoretic visualization with ethidium-bromide. All strains of PPV were detected from multiple hosts and from multiple Prunus tissues (leaf, stem, bud, and root). A dilution series using an in vitro synthesized transcript containing the target sequence as a standard demonstrated that the assay was effective for quantification of viral template. The assay was used to measure the levels of virus acquired by several aphid species.

INTRODUCTION
Plum pox potyvirus (PPV) is responsible for the disease shakka, considered the most economically destructive virus disease of cultivated Prunus (Lopez-Moya et al., 2000). PPV occurs in numerous economically important Prunus hosts, including peach, plum, apricot, nectarine, almond, and sweet and sour cherries. Multiple strains of PPV have been identified, including the most common D strain, the El Amar (EA) strain, the M strain and the cherry strain (C). PPV and shakka spread steadily throughout Europe from its origin, eventually reaching the middle-east, northern Africa, India, Chile, U. S., and Canada. Although eradication efforts have succeeded in rare instances in Europe, the overall course of the disease in Europe indicates that PPV moves and evolves effectively (Gottwald et al., 1995, Lopez-Moya et al., 2000).

PPV is a member of the genus Potyvirus in the family Potyviridae. The genome consists of a 9.7 kb, positive sense, ssRNA molecule, expressed as a 350 KDa polyprotein precursor that is posttranslationally processed by self-encoded proteases into 9 smaller functional proteins (Revers et al., 1999). PPV has been detected using a variety of biological, serological, and molecular methods (for review see Lopez-Moya, 2000). Polyclonal and monoclonal antibodies have been raised against all strains of PPV. Enzyme-linked immunosorbent assay (ELISA) remains the most common survey detection tool. However, strain variability, low virus titers, and uneven distribution of the virus in infected plants lead to detection inconsistencies (Nemeth, 1980). Viral titer can fluctuate greatly with host species, the age of the tree and the time of year (Polak, 1998). In addition, cross-reactivity with other potyviruses has been a problem with polyclonal antisera (Lopez-Moya, 2000).

PPV has been successfully detected using traditional PCR (Wetzell et al., 1991; Levy and Hadidi, 1994; Candresse et al., 1995; Faggioni et al., 1998), immuno-capture PCR (Wetzell et al., 1992), silica capture PCR (Malinowski, 1997) and print-capture PCR (Olmos et al., 1996). Despite the fact that all of these techniques are quite successful in detecting low titers of the virus, none of these protocols were quantitative in nature. Real-time PCR is an adaptation of the traditional PCR protocol that allows for the rapid detection of target-specific amplicons without post-PCR electrophoresis (Schaad et al., 2003). In addition, real-time PCR allows for the accurate quantification of target when used with a standard curve. PCR primer and probe sequences were selected for conserved regions of the PPV genome, and a PPV real-time PCR assay was developed and tested.

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The assay proved to be highly sensitive and very specific to PPV. In addition, the real-time format allows for very accurate quantification of the target RNA. This assay has been used to quantify PPV acquisition by aphid vectors and non-vectors.

MATERIALS AND METHODS
Pennsylvania isolates of PPV (PPV-D strain) were harvested from field sites, and PPV strains D, EA, M and C were brought in from Europe. Infected materials were maintained in a BSL-3 containment greenhouse. Total RNA was extracted from aphid or leaf tissue for use as a template. Primer and probe sequences were selected by determining suitable conserved regions in the PPV genome using multiple sequence alignments. To select the primers and the probe for the assay all current PPV sequences available in Genbank, including the D, M, C and EA strains, were aligned using the programPILEUP from the Genetics Computer Group (GCC) sequence analysis software package (version 9.0, Genetics Computer Group, Madison, WI) (Deveraux et al., 1984). The target region (Fig. 1) was selected from a conserved region in the middle of the coat protein (CP) gene (nucleotides 9198-9229). Nucleotide positions correspond to the reported sequence for PPV D, Genbank accession number X16415 (Treycheney et al., 1989). Probes (synthesized by Applied Biosystems Inc., Foster City, CA,USA) were labeled at the 5' end with the fluorescent reporter dye 6-carboxy-flourescin (FAM) and labeled at the 3' end with the quencher dye 6-carboxy-tetramethyl-rhodamine (TAMRA). The assay sensitivity was quantified using in vitro transcripts from a PPV clone.

The SuperScript One-Step RT-PCR with Platinum Taq kit (Invitrogen) was used throughout. The RT-PCR mixture contained 1X Reaction Mix (0.5 mM each of dATP, dCTP, dGTP, and dTTP, 1.2 mM MgSO4) additional 4.8 mM MgSO4, 200 nM forward and reverse primers, 100 nM FAM-TAMRA probe, and 0.5 μl RT/Platinum Taq Mix per reaction. Thermal cycling conditions were 52°C for 15 minutes for reverse transcription, 95°C for 5 minutes for Platinum Taq activation, and 60 cycles of 95°C for 15 seconds and 60°C for 30 seconds for PCR. Between 200 ng and 2 μg of total RNA or DNA template was used per assay. The cycle threshold (Ct) values for each reaction were calculated automatically by the Smart Cycler (Cepheid, Sunnyvale, CA,USA) detection software by determining the point in time (PCR cycle number) at which the reporter fluorescence exceeded background. Lower Ct values indicate higher quantities of target. The size of the PCR product was checked using electrophoresis.

Four species of aphids were allowed to feed on symptomatic pea leaves for two hours. As a control additional aphids from each species were allowed to feed on healthy leaves. After two hours the aphids were harvested in the following combinations: 50 aphids from infected plants; 10 aphids from infected plants plus 40 aphids from healthy; 1 aphid from infected tissue plus 49 from healthy; 50 aphids from healthy tissue. The aphids in tubes were flash frozen in liquid nitrogen, and total nucleic acid was extracted. Extracted total nucleic acid preps were tested using the real-time assay as described.

RESULTS AND DISCUSSION
Following confirmation of effective amplification using a DNA template, the real-time PCR assay was combined with reverse-transcription and tested on total RNA from healthy control and PPV- with infected peach trees and Colombo peas. No signal was detected using total RNA from healthy control plants. The PCR product generated by the CP primers was determined to be the expected size by electrophoresis (data not shown). Using the CP primer/probe set, the RT-PCR assay successfully detected all Pennsylvania isolates tested as well as the four known strains of PPV from Europe (data not shown). The assay did not detect any other potyviruses tested, nor did it detect other common insect vectors present from Prunus. The assay successfully detected PPV in all hosts tested (see Damsteegt et al., this issue). In addition, the assay detected PPV in a number of herbaceous hosts including peas, Nicotiana benthamiana, N. occidentalis, N. edwardsii, and Mollusus officinalis. No signal was detected in healthy control plants of any of these species. The assay detected PPV in all types of Prunus tissue, including
buds, leaves, flowers, fruit, roots, young stems (less than one year old growth) and woody stems. In addition to potential uses for diagnostics, the real-time PCR protocol described serves as a helpful tool for quantifying levels of viral RNA. In order to determine the lower detection limits and the quantitative ability of the assay in vitro, transcripts were synthesized from a 1.4 kb PPV clone that contained the target region of the CP. The assay consistently detected femtogram (fg) levels of an in vitro synthesized PPV transcript in a healthy plant sap background (Fig. 2). This level of sensitivity allows the assay to detect virus in combined or batched samples, where tissue from one infected leaf is included with tissue from four healthy leaves (data not shown). The assay was repeated four times to assess variability. Regression analysis of the four independent standard curves demonstrates that the assay is highly repeatable indicating the assay can be used with a standard curve to accurately estimate viral titer (data not shown).

Four species of aphids (3 vectors and 1 non-vector) were selected to test whether the real-time assay could quantify viral loads accumulated in vectors compared to non-vectors. For each aphid species, roughly 100 aphids were starved and then allowed to feed on symptomatic infected tissue for 2 hours. The aphids were removed from leaves and separated into microfuge tubes. Fifty aphids from infected leaves were put into one tube, ten aphids from infected leaves were combined with forty aphids from healthy leaves in a second tube, one aphid from an infected leaf was combined with forty-nine aphids in a third tube. Fifty aphids fed on healthy leaves were used as a negative control. The real-time assay detected virus in the fifty infected leaf aphid sample for Myzus persicae and Aphis spiraeola. Interestingly the Ct values indicate that M. persicae acquires the most virus. Comparing the results of the assay to the curve established using PPV transcripts indicates that M. persicae acquires at least 10 times as much virus as the other vector species Brachycyclus persicae and A. spiraeola. It is also interesting to note that the non-vector Acrithosiphon pismum accumulated significantly less virus than the vector species. Single aphid assays also were conducted for the four species. The assay was able to detect virus in single Myzus persicae and A. spiraeola, but not consistently. For example, the assay detected virus in one of three individual A. spiraeola, and two of nine M. persicae. Knowing that the assay is capable of detecting femtogram and occasionally attogram levels of viral RNA, we suspect that the inconsistent detection of virus in single aphids is due to inconsistency in the assay itself. In all likelihood this is an indication that only a percentage of an aphid population feeding on a leaf actually acquires virus, although this remains to be tested.

Literature Cited


Tables

Table 1. Quantifying PPV acquisition in aphid species. 50 aphids column represents samples where all aphids fed on infected tissue, 10 aphids column represents sample where 10 aphids fed on infected tissue were combined with 40 aphids that fed on healthy tissue, healthy aphid samples were fed on non-infected tissue.

<table>
<thead>
<tr>
<th>Aphid Species</th>
<th>Vector</th>
<th>50 aphids C&lt;sub&gt;T&lt;/sub&gt; value</th>
<th>10 aphids C&lt;sub&gt;T&lt;/sub&gt; value</th>
<th>Healthy aphids</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myzus persicae</td>
<td>Yes</td>
<td>20.45</td>
<td>26.35</td>
<td>Negative</td>
</tr>
<tr>
<td>Aphis epirocalca</td>
<td>Yes</td>
<td>27.45</td>
<td>20.88</td>
<td>Negative</td>
</tr>
<tr>
<td>Bradyucyndus persicae</td>
<td>Yes</td>
<td>28.03</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>Acyrhostiphon pustum</td>
<td>No</td>
<td>30.59</td>
<td>Negative</td>
<td></td>
</tr>
</tbody>
</table>

*C<sub>T</sub> values are the average of three experiments*
Fig. 1. 131 nucleotide region amplified by PPV CP forward and reverse primers. Probe sequence is identified on the negative sense (lower) strand.

Fig. 2. Quantification of PPV transcript using real-time PCR. The display shows the results from a typical assay where known quantities (2 fg to 2 ng) of a PPV transcript containing the target region were diluted in total RNA from healthy plants. In other experiments the assay has been able to detect as little as 500 aitograms of the PPV transcript.