SHORT COMMUNICATION

MONITORING OF PLUM BARK NECROSIS STEM PITTING-ASSOCIATED VIRUS AND MOLECULAR CHARACTERIZATION OF SOME ISOLATES

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SUMMARY

Different Prunus species from Italy, Serbia, Turkey and Egypt were assayed by one-step RT-PCR and nested PCR for the presence of Plum bark necrosis stem pitting-associated virus (PBNSPaV). This virus had a fairly high incidence and was monitored throughout the year by IC-RT-PCR and ELISA. It was detected during all seasons by both techniques but most easily in spring in the new flushes of vegetation. Six PBNSPaV isolates were characterized by partial sequencing of three genomic regions, e.g. HSP70, coat protein and ORF4. High nucleotide similarity was found between all isolates from different geographic and host origins, suggesting either a relatively recent origin or the presence of unidentified constraint on variation.

Key words: PBNSPaV, stone fruits, diagnosis, tracking, sequencing.

Plum bark necrosis stem pitting-associated virus (PBNSPaV), a member of the genus Ampelovirus, family Closteroviridae (Martelli et al., 2005; Al Rwahnih et al., 2007) with filamentous particles ca. 1500 nm in length (Amenduni et al., 2005), causes bark necrosis and gummosis on scaffold branches and the main trunk, and severe pitting of the woody cylinder on sensitive stone fruit cultivars (Marini et al., 2002; Boscia et al., 2010). PBNSPaV was first reported from the USA (Uyemoto and Teviotdale, 1996), later from Italy (Abou Ghanem-Ka¨n[System]azovic et al., 2001), Morocco (Bouani et al., 2004), Serbia (Mandic et al., 2005), Jordan (Sanchez-Navarro et al., 2005), Egypt (El Maghraby et al., 2006), Turkey (Usta et al., 2007) and France (Marais et al., 2009). The virus has a natural host range within the genus Prunus, its known hosts being Japanese and European plums, apricot, almond, peach, sweet and sour cherry (Marini, 1999; Abou Ghanem-Sabanadzovic et al., 2001; Bouani et al., 2004; Amenduni et al., 2005; Usta et al., 2007; Mandic et al., 2007).

The virus is not mechanically transmissible to herbaceous hosts (Abou Ghanem-Sabanadzovic et al., 2001) but can be detected by grafting to woody indicators. In the plum cvs Black Beaut, Angeleno and Friar, gummosis and bark necrosis begin in the second season after inoculation. In cherry cv. Colt, chlorotic ring spots on young leaves appear after 6 months, and light green or water-soaked marks along the leaf midrib in the second season. Leaf symptoms on GF305 peach, and Casselman, Laroda and Shiro plums consist of chlorotic rings and mottling, and line patterns (Marini et al., 2002). Serological methods using polyclonal antisera have been developed more recently (Boscia et al., 2006). Other rapid and more sensitive detection methods are RT-PCR-based assays (Abou Ghanem-Sabanadzovic et al., 2001), followed by nested PCR for improving sensitivity (Amenduni et al., 2005). Real-time RT-PCR and one-step RT-PCR have also been developed for virus detection (Al Rwahnih et al., 2007).

The present investigation focused on PBNSPaV monitoring throughout the year, and the identification and molecular characterization of virus isolates, since there are only a few PBNSPaV sequences available in GenBank.

The PBNSPaV-ASP isolate from apricot cv. Tiryinos (F1P27), from Apulia (southern Italy) (Abou Ghanem-Sabanadzovic et al., 2001) was graft-inoculated to one-year-old Black Beaut indicator in eight replicates in different combinations of rootstocks and interstocks (data not shown). The inoculated trees were grown in the experimental farm of the Faculty of Agriculture of the University of Bari. Symptoms were observed during the fifth year after grafting.

One of the field-grown tree grafted with isolate PBNSPaV-ASP was tested throughout the year, taking 18 leaf samples in spring (April), summer (July) and autumn (September), and 18 cortical tissue samples from dormant branches during winter (December). Samples were collected in the basal, central and apical part of
the tree canopy, and tested by ELISA and IC-RT-PCR. ELISA was done as described by Clark and Adams (1977), using a locally raised antiserum. IC-RT-PCR was performed using the ASP1 and ASP2 primers designed on the HSP70h gene sequence (Abou Ghanem-Sabanadzovic et al., 2001) and the protocol described by Wetzel et al. (1992).

A search was also made for natural PBNSPaV infections in randomly collected stone fruit samples from Italy, Serbia, Turkey and Egypt (Table 1). Total nucleic acid (TNA) was extracted according to Foissac et al. (2001). One-step RT-PCR was used for virus detection with SuperScript III RT/platinum Taq kit (Invitrogen, USA) according to the manufacturer's instructions using the above mentioned primers. Cycling conditions were 50°C for 30 min for cDNA synthesis, followed by 35 cycles at 94°C for 15 sec, 56°C for 30 sec and 68°C for 1 min. PCR products were visualized on 1.2% agarose/1x TAE gel stained with ethidium bromide.

Six PBNSPaV isolates were characterized in three parts of the genome (portions of the HSP70, CP, and ORF4 genes) by one-step RT-PCR (Table 2). Beside the ASP1 and ASP2 primers used for the HSP70h region, forward primer PBCPF (5'-TCACTCTTG-GTTCACGTG GCA-3') and reverse primer PBCPR (5'-CCACCTGTCGGACAGCTTCTT-3') were used for amplification of part of ORF4 and the CP region corresponding to nts 12,153-12,578, and forward primer PBP46F (5'-CTTACACGGTTGACAGATCTG-3') and reverse primer PBP46R (5'-CTCGCCTCAAAAGTTGTAGTC-3') for amplification of part of ORF4 corresponding to nts 11,040-11,469 (M. Al Rwahnih, unpublished information) [nt positions are determined from the full-length sequence of the isolate PL186 of PBNSPaV (GenBank accession No. EF546442)].

Because of difficulties in amplification of viral isolates TRPUN and SRBCUN with primers ASP1/ASP2, and PBCPF/PBCPR, nested PCR was used after one-step RT-PCR as described by Ameduni et al. (2005). Internal ASPn1 and ASPn2 primers were used for the HSP70h region (Ameduni et al., 2005), whereas forward primer PBCPn1 (5'-GAACGG TGCGTC-CAATCAGC-3') and reverse primer PBCPn2 (5'-CAGGAAAGTTGACACGCTTG-3') were designed for the ORF4/CP region corresponding to nts 12,241-12,424. One-step or nested PCR products were purified with the QiAquick PCR purification kit (Qiagen, Germany) and cloned into a pDrive vector (Qiagen, Germany) following standard procedures (Sambrook et al., 1989). DNA clones were custom sequenced by PRIMM (Italy). Multiple nucleotide and amino acid sequences were aligned with the program CLUSTAL X v. 1.81 (Thompson et al., 1997). Phylogenetic analysis was done using the version 2.1 of the Molecular Evolutionary Genetics Analysis (MEGA) software (Kumar et al., 2001).

The majority of Black Beaut plants graft-inoculated with isolate PBNSPaV-ASP showed severe symptoms of stem pitting (necrotic on some branches), bark necrosis and gumming, observed 5 years after grafting. Fruits were apparently normal but fewer than in healthy plants. During autumn, the leaves showed premature reddening.

PBNSPaV was found in different stone fruit samples with incidence of 26% in Italy, 40% in Egypt, 51% in Serbia and 70% in Turkey (Table 1). While there were no previous data from Italy and Serbia, the infection level detected in Turkey (70%) tallied with the 77% previously reported by Usta et al. (2007), and a higher incidence was found in Egypt (40%) compared with

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**Table 1.** Detection of PBNSPaV by one-step RT-PCR and nested PCR.

<table>
<thead>
<tr>
<th>Country</th>
<th>Tested samples (No.)</th>
<th>Plum (No.)</th>
<th>Cherry (No.)</th>
<th>Peach (No.)</th>
<th>Almond (No.)</th>
<th>Apricot (No.)</th>
<th>Total (No.)</th>
<th>Infection rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Italy</td>
<td>38</td>
<td>2</td>
<td>6</td>
<td>1</td>
<td>1</td>
<td>10</td>
<td>10</td>
<td>26</td>
</tr>
<tr>
<td>Serbia</td>
<td>43</td>
<td>22</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>22</td>
<td>22</td>
<td>51</td>
</tr>
<tr>
<td>Turkey</td>
<td>20</td>
<td>-</td>
<td>-</td>
<td>14</td>
<td>-</td>
<td>14</td>
<td>14</td>
<td>70</td>
</tr>
<tr>
<td>Egypt</td>
<td>15</td>
<td>6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>6</td>
<td>6</td>
<td>40</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>116</strong></td>
<td><strong>8</strong></td>
<td><strong>22</strong></td>
<td><strong>20</strong></td>
<td><strong>1</strong></td>
<td><strong>1</strong></td>
<td><strong>52</strong></td>
<td></td>
</tr>
</tbody>
</table>

**Table 2.** PBNSPaV isolates used for molecular characterization.

<table>
<thead>
<tr>
<th></th>
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<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Japanese plum</td>
<td>Santa Rosa</td>
<td>Egypt</td>
<td>EGSA   NO.</td>
<td>GU079638</td>
<td>GU079647</td>
<td>GU079645</td>
</tr>
<tr>
<td>2</td>
<td>Japanese plum</td>
<td>Unknown</td>
<td>Italy</td>
<td>ITPUN</td>
<td>GU079637</td>
<td>GU079648</td>
<td>GU079643</td>
</tr>
<tr>
<td>3</td>
<td>European plum</td>
<td>Cuore Borgia</td>
<td>Italy</td>
<td>ITBOR</td>
<td>GU079636</td>
<td>GU079649</td>
<td>GU079644</td>
</tr>
<tr>
<td>4</td>
<td>Apricot</td>
<td>Tyrinhos</td>
<td>Italy</td>
<td>ITATYR N.S.</td>
<td>GU079650</td>
<td>GU079650</td>
<td>GU079646</td>
</tr>
<tr>
<td>5</td>
<td>Cherry</td>
<td>Unknown</td>
<td>Serbia</td>
<td>SRBCUN</td>
<td>GU079640</td>
<td>GU079652</td>
<td>GU079642</td>
</tr>
<tr>
<td>6</td>
<td>Peach</td>
<td>Unknown</td>
<td>Turkey</td>
<td>TRPUN</td>
<td>GU079639</td>
<td>GU079651</td>
<td>GU079641</td>
</tr>
</tbody>
</table>

*N.S. = not sequenced, the sequence already present in GenBank (accession No. AJ305307)
21% reported by El Maghraby et al. (2007).

Besides the choice of diagnostic technique and testing period, the sampling method and type of samples collected were crucial for the reliability of the assays. Since this kind of information was limited for PBNSPaV, leaf and cortical tissues, collected from the quadrant of a plum tree graft-inoculated with isolate ASP, were tested by ELISA and IC-RT-PCR at seasonal intervals during the year. The results are summarized in Table 3.

The virus was detected by ELISA in young leaves collected in the spring from the tree infected by grafting (100%). From dormant branches in winter, and leaves in summer, detection was 94%, and 83% respectively. The lowest detection rate was from mature leaves in autumn (56%). These results fit with the data obtained by Boscia et al. (2006), confirming that spring leaves are the best for reliable detection by ELISA. IC-RT-PCR detection was highly positive (100%) from all samples in spring, summer and autumn and a bit lower (89%) in winter. Considering the results over all seasons, both techniques gave satisfactory detection, since IC-RT-PCR detected the virus in 97% and ELISA in 83% of the samples.

The average detection by both techniques was higher in the basal and central parts of the branches (92%), than in the apical parts (88%). In particular, IC-RT-PCR detection was positive from all samples taken from basal parts of twigs in all seasons, and 96% of the samples collected from central and apical parts of twig. ELISA gave the highest detection rate in samples coming from the central parts of the branches (88%), followed by 83% in the basal and 79% in apical parts. Considering that these data came from a single plum tree, confirmatory results from other host species and environmental conditions appear desirable.

Six PBNSPaV isolates were selected to study molecular variability in the three genomic regions (Table 2). Five of these isolates were from naturally infected plants, while isolate ITATYR originated from an apricot grafted on Black Beaut, whose HSP70h gene had been previously sequenced only in part [accession No. AJ305307, Abou Ghanem-Sabanadzovic et al. (2001)]. The sequenced PCR products had sizes of 240 bp (HSP70h), 242 bp (CP) and 238 bp (ORF4). Nested PCR products were sequenced only for isolates SRBCUN and TRPUN (145 bp of the HSP70h and 92 bp of the CP genes).

Newly sequenced and previously published (accession Nos. EF546442, AF195501, AJ305307, and FJ231498) sequences of a HSP70h fragment of different virus isolates were compared at the nucleotide and amino acid level showing a similarity ranging from 92 to 100%. The most distant was the previously published isolate Malatya K1 from Turkey (accession No. FJ231498), that showed three amino acid changes: S to P, V to F, and T to S at amino acid positions 96, 110,
and 125, respectively (numbers refer to the amino acid positions in the full-length sequence of PL186 isolate under accession No. EF546442). The newly sequenced isolate TRPUN, also originating from Turkey, showed two changes in contiguous amino acids: R to P, and V to L at amino acid positions 106 and 107, respectively.

Phylogenetic analysis of the sequenced part of ORF4 gave two different clusters: one containing all Italian isolates and the American isolate PL186, another containing isolates from Egypt, Serbia and Turkey (EGSAN, SRBCUN, and TRPUN, respectively). EGSAN, SRBCUN, and TRPUN isolates showed V to A change in position 195, and V to I in position 254. Three other isolates had the same amino acid sequences as PL186 in the sequenced portion of the ORF4 gene. Two clusters were obtained by analysis of the sequenced fragment of the CP gene, one of which again comprised isolates SRBCUN, TRPUN, and EGSAN plus two other isolates (ITBOR and ITATYR), while the other contained isolates PL186 and ITIPUN (data not shown).

There was no correlation between sequence and host from which the virus isolate derived, nor a clear grouping on the basis of geographic origin. Considering that the results of phylogenetic analysis were obtained from only few isolates, sequencing of a larger number of isolates should be done to confirm our results. Also, it should be noted that only plum isolates were easily detected by one-step RT-PCR; all other stone fruit isolates required amplification in some parts of the genome through nested PCR for reliable identification. Our results showed that the non-plum PBNSPaV isolates had high sequence homology with plum isolates, so the need for nested PCR was probably due to low viral titer rather than sequence variability. This was confirmed during the field survey when non-plum isolates were mainly detected only by nested PCR (data not shown).

The present study has shown the high molecular homology of six PBNSPaV isolates from different geographic and host origins. Nucleotide and amino acid sequence alignments revealed a relatively high level of homology (more than 90%) in three genomic regions, most sequences being more than 95% homologous. The high molecular similarity of isolates from different countries and different stone fruit species could be interpreted as being consequent to a recent origin of the virus population or to the involvement of constraining factors that reduce molecular variability, such as, for example, transmission by a vector which has not been identified although natural virus spread has been reported (Di Terlizzi and Savino, 1995).

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