Nucleotide Sequence and Structural Features of Hop Stunt Viroid and Citrus Bent Leaf Viroid Variants from Blighted Citrus Plants in Kohgiluyeh–Boyerahmad Province of Iran

M. Amiri Mazhar1, S. A. A. Bagherian1,2, A. Salahi Ardakani3, and K. Izadpanah4

ABSTRACT

Hop stunt viroid (HSVd) isolates have been reported as the causal agent of citrus cachexia in Mazandaran Province and recently shown to be associated with yellow corky vein disease of sweet orange and split bark disorder of sweet lime in the Fars Province, Iran. In the present work isolation and partial characterization of viroids from citrus trees affected by gummy stem blight is reported from Kohgiluyeh–Boyerahmad (KB) Province of Iran. Fifteen samples of citrus trees from Dehdasht area (KB Province) showing bark necrosis, gum exudation and die-back as well as seven citrus symptomless trees from the same area were tested for the prevalence of viroids, through Reverse Transcription Polymerase Chain Reaction (RT-PCR) followed by sequencing of PCR products. They were also tested for Citrus tristeza virus through Double-Antibody Sandwich Enzyme Linked Immunosorbtent Assay (DAS-ELISA). Two variants of HSVd which differed from GenBank isolates in nucleotide sequence and two variants of Citrus Bent Leaf Viroid (CBLVd) were identified in any of the symptomatic samples. Moreover, a Citrus Exocortis Viroid (CEVd) was found only in symptomatic sweet lime. An HSVd isolate from KB (HSVd-bn1) was selected and used for comparison with a number of HSVd variants from Iran (Fars and Mazandaran Provinces) and the related accessions from GenBank. On the basis of nucleotide sequence and secondary structure analysis, HSVd-bn1 and HSVd-bn2 belong to non-cachexia variants of HSVd and have about 95% similarity to Citrus gummy bark viroid, a sub-species of HSVd. CTV was not detected in the diseased plants. It is yet to be determined whether bark necrosis of sweet lime and of sweet orange plants is caused solely by the associated viroid(s) or other factors are involved as well.

Keywords: Citrus bent leaf viroid, Citrus gummy bark, Hop stunt viroid, Phylogeny.

INTRODUCTION

Citrus Exocortis Viroid (CEVd) and Hop stunt viroid (HSVd) have been shown to cause distinct diseases of citrus, i.e., exocortis and cachexia, respectively (Semancik et al., 1988; Reanwarakorn and Semancik, 1999). However, HSVd has been found in a wide range of other hosts including hop, cucumber, grapevine, plum, peach, pear, apricot and almond (Ohno et al., 1983; Shikata, 1990; Astruc et al., 1996; Cañizares et al., 1999). In some hosts, the infection by HSVd is associated with such serious disorders of economic importance as stunting of hop (Shikata, 1990), dapple fruit disease of plum and peach (Sano et al., 1989; Ragozzino et al., 2002) and cachexia of citrus (Diener et al., 1988; Semancik et al., 1988), yellow corky vein disease of sweet orange and split bark of sweet lime (Bagherian and Izadpanah, 2010). Citrus
viroid-II (CVd-II) (a synonym for HSVd) includes the non-cachexia variants (CVd-IIa) and the causal agents of mild (Ca902) and severe cachexia (CVd-IIb, CVd-IIc) (Banihashemian et al., 2010).

Recently, a new disease of citrus characterized by bark necrosis and decline has appeared in the Kohgiluyeh–Boyerahmad (KB) Province of Iran. This paper describes the disease and reports on characterization of HSVd, CEVd and Citrus Bent Leaf Viroid (CBLVd) detected in the affected plants.

MATERIALS AND METHODS

Five sweet lime (Citrus limettoides) and ten sweet orange (Citrus sinensis) trees showing stem blight symptoms vs. seven symptomless trees (4 sweet orange and 3 mandarin, Citrus reticulata trees) were sampled in Dehdasht area in the KB province of Iran in 2010-2012. Extraction of nucleic acids from either leaf veins or shoot bark was carried out through phenol-chloroform RNA extraction protocol, designed to yield high viroid titers (Semancik et al., 1975). A citrus sample known to be infected with HSVd (Bagherian and Izadpanah, 2010) was used as the positive control. The negative control consisted of leaf samples of non-symptomatic citrus. Double Antibody Sandwich Enzyme Linked Immunosorbent Assay (DAS-ELISA) was performed to examine the samples for the presence of Citrus Tristeza Virus (CTV) (Bar-Joseph et al. 1979) making use of a diagnostic kit (AgriTest, Italy).

Inoculation of viroids to herbaceous plants was performed through injection of total nucleic acid (Fei et al., 2009) obtained from leaf veins of a sweet lime tree showing bark necrosis into the stems of four cucumber and four tomato plants. Inoculated plants were kept in the greenhouse with the infectivity test being performed 30 days postinoculation using RT-PCR.

RT-PCR analyses were performed to detect CEVd, CBLVd, HSVd, Citrus Viroid-III (CVd-III), Citrus Viroid-IV (CVd-IV) and Citrus Viroid-V (CVd-V) following the method described by Bernad and Duran-Vila (2006) using primer pairs designed to amplify the full length of each viroid (Table 1).

Samples were denatured at 95°C for 5 minutes. First-strand viroid cDNA was synthesized with 200 U of Moloney Murine Leukemia Virus Reverse Transcriptase (MMuLV-RT) (Fermentas, Lithuania) using the specific reverse primer (1 µM), dNTPs (1 mM each) and 4 µl of 5X reaction buffer (250 mM Tris–HCl (pH 8.3), 20 mM MgCl₂, 250 mM KCl and 50 mM DTT). The reaction mixture was adjusted to 20 µl with deionized H₂O and incubated at 42°C for 1 minute.

Table 1. Citrus viroid specific primers used in RT-PCR.

<table>
<thead>
<tr>
<th>Viroid</th>
<th>Direction</th>
<th>Primer Sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEVd</td>
<td>Reverse</td>
<td>5′-CCGCGGATCCCTGAAGGA-3′</td>
<td>Gross et al. (1982)</td>
</tr>
<tr>
<td>HSVd</td>
<td>Reverse</td>
<td>5′-GGAAACCTGGAGGAAGTCG-3′</td>
<td>Sano et al. (1988)</td>
</tr>
<tr>
<td>CBLVd</td>
<td>Reverse</td>
<td>5′-GGGGCAACTCTTCTCAAGAATCC-3′</td>
<td>Ashulin et al. (1991)</td>
</tr>
<tr>
<td>CVd-III</td>
<td>Reverse</td>
<td>5′-TCGACGACGACCAGTCAGCT-3′</td>
<td>Bernad and Duran-Vila (2006)</td>
</tr>
<tr>
<td>CVd-IV</td>
<td>Reverse</td>
<td>5′-GGGGCAACTCTTCTCAAGAATCC-3′</td>
<td>Bernad and Duran-Vila (2006)</td>
</tr>
<tr>
<td>CVd-V</td>
<td>Reverse</td>
<td>5′-GGAACCACAAGGTTGTTCAC-3′</td>
<td>Serra et al. (2007)</td>
</tr>
</tbody>
</table>

a Citrus Exocortis Viroid, b Hop stunt viroid, c Citrus bent leaf viroid, d Citrus viroid III, e Citrus viroid IV, f Citrus viroid V.
hour. Second-strand cDNA synthesis and PCR amplification were performed in 50 µl final volumes using 4 µl of the first-strand cDNA reaction mixture, 1 U Taq DNA polymerase (CinnaGen), the selected forward and reverse primer pair (Table 1) (0.5 µM each) and dNTPs (0.12 mM each) in a buffer containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl and 1 mM MgCl₂. PCR parameters consisted of a denaturation step at 94°C for 5 minutes, followed by 35 cycles of 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 1 minute and finished with an extension step at 72°C for 5 minutes. PCR products were electrophoresed in 1% agarose gel and purified from gel using the AccuPrep® PCR Purification Kit (Bioneer, Korea).

Purified RT-PCR products of all positive samples were sequenced by Tech Dragon Inc., Hong Kong. These sequences were compared against GenBank using NCBI/Basic Local Alignment Search Tool (BLAST) engine. Alignment of sequences was performed using the Vector NTI 9 software package (InforMax, Bethesda, MD).

Phylogenetic analysis was carried out employing DNAMAN software (version 4.0.1.1) with a tree constructed, using the neighbor-joining method (Saitou and Nei, 1987) based on 10,000 bootstrap replicates. Genetic distances were assessed, making use of the MegAlign program in the DNASTAR software package (Madison, WI). The most stable secondary structure analyses were obtained with the RNAstructure software (version 4.6). The nucleotide sequence data reported in this paper were submitted to the EMBL, GenBank and DDBJ nucleotide sequence databases.

RESULTS AND DISCUSSION

Gummy stem blight was found in a large proportion of sweet lime and sweet orange grafted on sour orange rootstocks in the KB province. It was characterized by bark necrosis above the graft union, gum impregnation and exudation from the affected areas, shoot blight, die back and finally a decline of the tree (Figure 1). The disease was observed in both old and young trees with the symptoms resembling those of certain other citrus disorders with, viroid association (Semancik et al., 1975; Onelge et al., 2004; BaniHashemian et al., 2010; Sofy et al., 2010).

RT-PCR amplifications using HSVd, CBLVd and CEVd specific primers produced amplicons of ~300, ~230 and ~370 bp, respectively (Figure 2). HSVd and CBLVd were detected in all the fifteen symptomatic samples while CEVd was detected only in the five symptomatic sweet lime samples. No amplification was obtained from the seven symptomless samples which were used as healthy control in RT-PCR. CvD-III, CvD-IV and CvD-V were not detected in any sample.

All the samples proved negative for CTV in DAS-ELISA with specific antiserum (data not shown).

The nucleotide sequence analyses of HSVd, CBLVd and CEVd exhibited two new variants of HSVd (designated HSVd-bn1 and HSVd-bn2) that differed in a single
nucleotide (position 58) in the pathogenicity domain. Of the nine HSVd samples sequenced, three were identical to bn1 while six belonging to bn2. All the 15 CBLVd sequences were identical except for the absence of a T at position 46 in 4 isolates (Acc. No. JQ080281). CEVd was found only in symptomatic sweet limes (Bagherian et al., 2009). The nucleotide sequences of the HSVd-bn1 and HSVd-bn2 were deposited in GenBank (Acc. Nos. JQ080278 and JQ080279).

Biological indexing showed leaf epinasty symptoms in all the tomato plants inoculated by extracts from a symptomatic sweet lime tree (Figure 3) whereas inoculated cucumber plants did not show any symptoms. Infectivity test 30 Days PostInoculation (DPI) revealed that both CEVd and HSVd replicated in all the inoculated tomato and cucumber plants (data not shown).

Sequence analysis revealed that HSVd-bn1 (JQ080278) and HSVd-bn2 (JQ080279) were both 302 nt long, 3 nt longer than CGBVd and 2 nt longer than HSVd-cit 5, but of the same length as HSVd-cit 6. A comparison of the secondary structure of HSVd-bn1 and HSVd-bn2 RNA (generated through RNA Structure software, version 4.6) with other viroid variants indicated their high similarities with respect to the rod-like structure, number of loops and free energy which were suggestive of a highly base paired, heat stable molecule, characteristic of viroid-like low molecular weight RNAs.

In Clustal analysis, sequences of HSVd-bn1 and HSVd-bn2 aligned with other HSVd variants showing nearly 100% sequence identity with HSVd-cit 6 (GQ246199) isolated from Clementine.
mandarin on Carrizo citrange (Banihashemian et al., 2010), but with comparatively lower homologies with many other HSVd variants including those associated with gummy and split bark and as well with yellow corky vein of citrus (Table 2).

Phylogenetic analysis revealed that HSVd-bn1 and HSVd-bn2 belonged to the same cluster as citrus gummy bark, sweet orange yellow corky vein and cucumber isolates. They formed a clade with HSVd-Cit 5 and HSVd-Cit 6 (Figure 4). Symptoms of gummy stem blight of sweet lime and sweet orange are similar to those of sweet orange gummy bark, first described by Nour-Eldin (1956), as a phloem discoloration and stunting of sweet orange. Another gummy bark disorder of sweet orange has been associated with Cv-d-II (Sofy et al., 2010), which is 100% identical to Cvd-Ib or Ca902 (AF131249) (Reanwarakorn and Semancik, 1999). *Citrus Gummy Bark Viroid* (CGBVd), is a variant of Cv-d-Ib and Cv-d-Iic which are considered as severe cachexia inducing variants. HSVd-bn1 was 96% identical to Cv-d-Iic with sequence variations in all the five structural domains of rod structure (Onelge et al., 2004; Sofy et al., 2010).

Nucleotide sequences of the HSVd-bn1 and HSVd-bn2 were nearly 100% similar to HSVd-cit 5 (Wang et al., 2010) and HSVd-bn2 was 100% similar to HSVd-cit 6. The latter, however, did not induce symptoms in Clementine grafted on Carrizo citrange in Spain (Banihashemian et al., 2010). On the basis of sequence analyses, these variants belong to Cv-d-IIa sub-group (non-cachexia inducing variants of HSVd). HSVd-bn1 and HSVd-bn2 differ from CGBVd in all the six nucleotides in the so-called “cachexia expression motif” in the variable domain. The “cachexia expression motif” plays an important role in the inciting symptoms so that changes within this motif affect

### Table 2. Percentage sequence identities of HSVd-bn1 with other viroid variants.

<table>
<thead>
<tr>
<th>Variant</th>
<th>Accession no.</th>
<th>Host</th>
<th>Reported from</th>
<th>Number of nucleotides</th>
<th>Percent identity with HSVd-bn1</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSVd-bn1*</td>
<td>JQ080278</td>
<td>Citrus</td>
<td>Iran</td>
<td>302</td>
<td>100</td>
</tr>
<tr>
<td>HSVd-bn2</td>
<td>JQ080279</td>
<td>Citrus</td>
<td>Iran</td>
<td>302</td>
<td>99.7</td>
</tr>
<tr>
<td>Citrus gummy bark viroid</td>
<td>FJ984562</td>
<td>sweet orange</td>
<td>Egypt</td>
<td>299</td>
<td>96.0</td>
</tr>
<tr>
<td>HSVd-cit 1</td>
<td>AF131249</td>
<td>Citrus</td>
<td>California</td>
<td>299</td>
<td>96.0</td>
</tr>
<tr>
<td>HSVd-cit 2</td>
<td>FJ716177</td>
<td>Citrus</td>
<td>China</td>
<td>300</td>
<td>99.0</td>
</tr>
<tr>
<td>HSVd-cit 3</td>
<td>EF126046</td>
<td>Citrus</td>
<td>Iran (Mazandaran)</td>
<td>300</td>
<td>99.7</td>
</tr>
<tr>
<td>HSVd-cit 4</td>
<td>EF186992</td>
<td>Citrus</td>
<td>Iran (Mazandaran)</td>
<td>300</td>
<td>99.3</td>
</tr>
<tr>
<td>HSVd-cit 5</td>
<td>FJ716209</td>
<td>Citrus</td>
<td>China</td>
<td>300</td>
<td>99.7</td>
</tr>
<tr>
<td>HSVd-cit 6</td>
<td>GQ246199</td>
<td>Citrus</td>
<td>Spain</td>
<td>302</td>
<td>99.7</td>
</tr>
<tr>
<td>HSVd-cit 7</td>
<td>X000009</td>
<td>Citrus</td>
<td>Japan</td>
<td>297</td>
<td>95.9</td>
</tr>
<tr>
<td>HSVd-cit 8</td>
<td>AB054615</td>
<td>Citrus</td>
<td>Spain</td>
<td>297</td>
<td>99</td>
</tr>
<tr>
<td>HSVd-cit 9</td>
<td>AF131248</td>
<td>Citrus</td>
<td>University of California-Riverside</td>
<td>302</td>
<td>99.3</td>
</tr>
<tr>
<td>HSVd-sb</td>
<td>FJ465507</td>
<td>Sweet lime</td>
<td>Iran (Fars)</td>
<td>299</td>
<td>94.3</td>
</tr>
<tr>
<td>HSVd-sycv</td>
<td>FJ465506</td>
<td>Sweet orange</td>
<td>Iran (Fars)</td>
<td>302</td>
<td>98.7</td>
</tr>
<tr>
<td>HSVd-alm</td>
<td>AJ011813</td>
<td>Almond</td>
<td>Spain</td>
<td>296</td>
<td>92.2</td>
</tr>
<tr>
<td>HSVd-apr</td>
<td>AJ297840</td>
<td>Prunus</td>
<td>Spain</td>
<td>297</td>
<td>93.6</td>
</tr>
<tr>
<td>HSVd-cuc</td>
<td>X00524</td>
<td>Cucumber</td>
<td>Japan</td>
<td>303</td>
<td>98.0</td>
</tr>
<tr>
<td>HSVd-gra</td>
<td>M35717</td>
<td>Grapevine</td>
<td>United States and Japan</td>
<td>296</td>
<td>95.9</td>
</tr>
<tr>
<td>HSVd-pea</td>
<td>D13765</td>
<td>Peach</td>
<td>Japan</td>
<td>297</td>
<td>95.3</td>
</tr>
<tr>
<td>HSVd-plu</td>
<td>D13764</td>
<td>Plum and Peach</td>
<td>Japan</td>
<td>297</td>
<td>92.2</td>
</tr>
<tr>
<td>CEVd b</td>
<td>J02053</td>
<td>Citrus</td>
<td>Iran</td>
<td>371</td>
<td>48.9</td>
</tr>
</tbody>
</table>

*a* Hop stunt viroid, *b* Citrus Exocortis Viroid
Figure 4. A phylogram drawn through neighbor joining bootstrap method in CLUSTAL X (1.81b) software, illustrating phylogenetic position of HSVd-bn1 and HSVd-bn-2 among the HSVd variants. See Table 2 for a specification of viroid variants.

Figure 5. A comparison of the cachexia expression motif of HSVd-bn (-1 and -2) and some other citrus variants of HSVd. Nucleotides: Non-homologous sequences; Nucleotides: Conserved sequences; Nucleotides: Homologous sequences, (-): Lack of nucleotide.

symptom severity and may even suppress symptom expression (Serra et al., 2008). HSVd-bn1 and HSVd-bn2 differ from HSVd-cit 4 in two nucleotides in this motif while no differences are detected between HSVd-bn1 and -2, HSVd-cit 5, HSVd-cit 6 and non-pathogenic isolate CVd-IIa-117 (AF213503) in cachexia expression motif (Figure 5). However CVd-IIa-117 complete sequence was of only about 98% identity with CGBVd and was only 95% identical to HSVd-bn1 and HSVd-bn2.

On the other hand HSVd-cit 4 has been reported to be associated with citrus cachexia in Mazandaran Province (Alavi et al., 2006). HSVd-bn1, HSVd-bn2, and HSVd-cit 4 from Mazandaran clustered in the same group, whereas other isolates from citrus in Fars Province were placed in different groups. This indicates the existence
of more variation in the genome of HSVd isolates in Fars than in Mazandaran and KB provinces. CBLVd-Iran was very similar to CBLVd-A (Acc. No.: FJ773267) and CBLVd-A33 (Acc. No.: FJ773265) from Pakistan (Punjab) with identities of 98.3 and 97.4%, respectively (Figure 6), suggesting that both viroids came from a common origin.

Bark cracking on Poncirus trifoliata rootstock has been attributed to a non-cachexia variant of HSVd (CVd-IIa). This condition does not result in economic damage to trees or crop but, on the contrary, sweet orange with CVd-IIa exhibits an enhancement in commercial performance (Onelge et al., 2004).

Although no other agents were found to be constantly associated with bark necrosis and gummy stem blight of citrus in KB province of Iran, it is yet to be determined whether the disease is caused by HSVd variants, a viroid complex (Verniere et al., 2006)) or such other factors as host and environmental conditions. Further work is needed to determine the factors involved in bark necrosis and stem blight of citrus in KB Province.

**REFERENCES**


**Figure 6.** Comparison among primary sequence of CBLVd from Iran and two other highly similar CBLVd variants. Sequences are aligned for maximum homology using Vector NTI program (version 9.0.0). Nucleotides: Homologous sequences; (-): Lack of nucleotide; Nucleotides: Non homologous sequences.


ترادف نوکلوتیدی و ویژگی‌های ساختاری واریانت های ویروئید کوتولنگی رازک
و ویروئید پیچیدگی برگ مربکت از استان کهگیلویه و بویراحمد

م. امیری مظهر، س. ع. ا. باقریان، ع. صلاحی اردکانی، و ک. ایزدبانی

چکیده

جدایی‌های های ویروئید کوتولنگی رازک (HSVd) به عضوی از کانکسی‌ای مربکت در مازندران
معرفی شده‌اند و اخیراً مشخص گردیده است که این ویروئید با بیماری زرد و چوب پنه‌ای شدن
رگی‌گرک پرتفاقد و عارضه شفافی می‌باشد. در استان فارس در ارتباط است. در این مطالعه ویروئیدهای
هموارا با دو رکه از مارکدارهای سنتی است. در استان کهگیلویه و بویراحمد
جداسازی و برخی ویژگی‌های آن‌ها گزارش گردیده است. درختان پرتفاقد و لیموفیرین پیوند شده
روی نارنج‌که دچار نکروز پوست و ترشح صمغ از تنه و ساقه و سرخ‌پیدی که در منطقه دهدشته
نمونه‌برداری شده و به کمک RT-PCR نمونه‌های های به روش ال‌پی‌آر و جهت ردایی و گروه ترسیتی نیز برسی شدند. در ویروئید HSVd و دو
واریانت ویروئید پیچیدگی برگ مربکت که با ترافی‌های های بالاک زن متوازی بودند. در تمام
نمونه‌های دارای علائم شناسایی گردید. یک واریانت ویروئید اگروکرین به مرکبات نش مفید
در لیموفیرین های دارای علائم سیگنال بود. این مطالعه چنین نشان داد که HSVd-1 و HSVd-bn2
نمونه‌های های از جمله HSVd-1 و HSVd-bn2 و از جمله HSVd-bn1

جریان بر اساس ترافی‌های نوکلوتیدی و آنالیزهای ساختار ناحیه‌ای، های ویروئید فارس و گرفته و 95 درصد تشخیص با بیماری ترشح صمغ به راه
نان دادن. از موارد دیگر، ویروس ترسیتی در گیاهان آلوه رنگ‌یابی نشده. هنوز پیشنهاد مشخص شود که
نکروز های لیموفیرین و پرتفاقد پوستی و ویروئید (HSVd) ایجاد می‌شود و یا یکی از عوامل نیز دخلا دارد.