J. Agr. Sci. Tech. (2014) Vol. 16: 657-665

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

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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 symptompless 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 Immunosorbent 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-bn$_1$) 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*

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[DOR: 20.1001.1.16807073.2014.16.3.18.6]

virodi-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 $MgCl_2$, 250 mM KCl and 50 mM DTT). The reaction mixture was adjusted to 20 µl with deionized H_2O and incubated at 42°C for 1

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

Viroid	Direction	Primer Sequence	Reference
CEVd [a]	Reverse	*5′-CCGGGGATCCCTGAAGGA-3′*	Gross *et al.* (1982)
	Forward	*5′-GGAAACCTGGAGGAAGTCG-3′*	
HSVd [b]	Reverse	*5′-GGGGCTCCTTTCTCAGGTAAGTC-3′*	Sano *et al.* (1988)
	Forward	*5′-GGGGCAACTCTTCTCAGAATCC-3′*	
CBLVd [c]	Reverse	*5′- TCGACGACGACCAGTCAGCT-3′*	Ashulin *et al.*(1991)
	Forward	*5′-TCCCCTTCACCCGAGCGCTGC-3′*	
CVd-III [d]	Reverse	*5′-TTCGTCGACGACGACAGGTA-3′*	Bernad and Duran-Vila (2006)
	Forward	*5′-GGCAGCTAAGTTGGTGACGC-3′*	
CVd-IV [e]	Reverse	*5′-GGGGATCCCTCTTCAGGT-3′*	Bernad and Duran-Vila (2006)
	Forward	*5′-GGGGAAATCTCTTCAGAC-3′*	
CVd-V [f]	Reverse	*5′-GGAACCACAAGGTTGTTCAC-3′*	Serra *et al.* (2007)
	Forward	*5′-TGTGGGTCACCCCGCCCC-3′*	

[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.

[DOR: 20.1001.1.16807073.2014.16.3.18.6]

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 T*aq* 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 $MgCl_2$. 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

Figure 1. Citrus tree affected by gummy stem blight.

[DOR: 20.1001.1.16807073.2014.16.3.18.6]

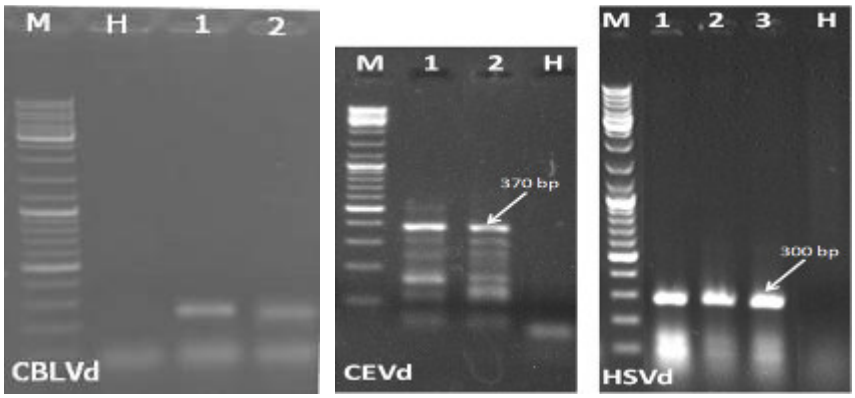

Figure 2. Electrophoretic patterns of RT-PCR products with CBLVd (left), CEVd (center) and HSVd (right) specific primers; diseased citrus samples showed specific bands at 230, 370, and 300 bp, respectively. (1: Positive control; 2 and 3: Infected samples; H: Healthy control, M: 100-bp ladder).

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 revealerd 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

Figure 3. Leaf epinasty in tomato plant inoculated with nucleic acid extract from sweet lime affected by bark necrosis.

[DOR: 20.1001.1.16807073.2014.16.3.18.6]

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 CVd-II (Sofy *et al.*, 2010), which is 100% identical to CVd-IIb or Ca902 (AF131249) (Reanwarakorn and Semancik, 1999). *Citrus Gummy Bark Viroid* (CGBVd), is a variant of CVd-IIb and CVd-IIc which are considered as severe cachexia inducing variants. HSVd-bn1 was 96% identitical to CVd-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-bn_2 was 100% similar to HSVd-cit 6. The latter, howener 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 CVd-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.

Variant	Accession no.	Host	Reported from	Number of nucleotides	Percent identity with HSVd-bn1
HSVdbn$_1$[a]	JQ080278	Citrus	Iran	302	100
HSVd-bn$_2$	JQ080279	Citrus	Iran	302	99.7
Citrus gummy bark viroid	FJ984562	sweet orange	Egypt	299	96.0
HSVd-cit 1	AF131249	Citrus	California	299	96.0
HSVd-cit 2	FJ716177	Citrus	China	300	99.0
HSVd-cit 3	EF126046	Citrus	Iran (Mazandaran)	300	99.7
HSVd-cit 4	EF186992	Citrus	Iran (Mazandaran)	300	99.3
HSVd-cit 5	FJ716209	Citrus	China	300	99.7
HSVd-cit 6	GQ246199	Citrus	Spain	302	99.7
HSVd-cit 7	X00009	Citrus	Japan	297	95.9
HSVd-cit 8	AB054615	Citrus	Spain	297	99
HSVd-cit 9	AF131248	Citrus	University of California-Riverside	302	99.3
HSVd-sb	FJ465507	Sweet lime	Iran (Fars)	299	94.3
HSVd-sycv	FJ465506	Sweet orange	Iran (Fars)	302	98.7
HSVd-alm	AJ011813	Almond	Spain	296	92.2
HSVd-apr	AJ297840	Prunus	Spain	297	93.6
HSVd-cuc	X00524	Cucumber	Japan	303	98.0
HSVd-gra	M35717	Grapevine	United States and Japan	296	95.9
HSVd-pea	D13765	Peach	Japan	297	95.3
HSVd-plu	D13764	Plum and Peach	Japan	297	92.2
CEVd [b] (Out group)	J02053	Citrus	Iran	371	48.9

[a] Hop stunt viroid, [b] Citrus Exocortis Viroid

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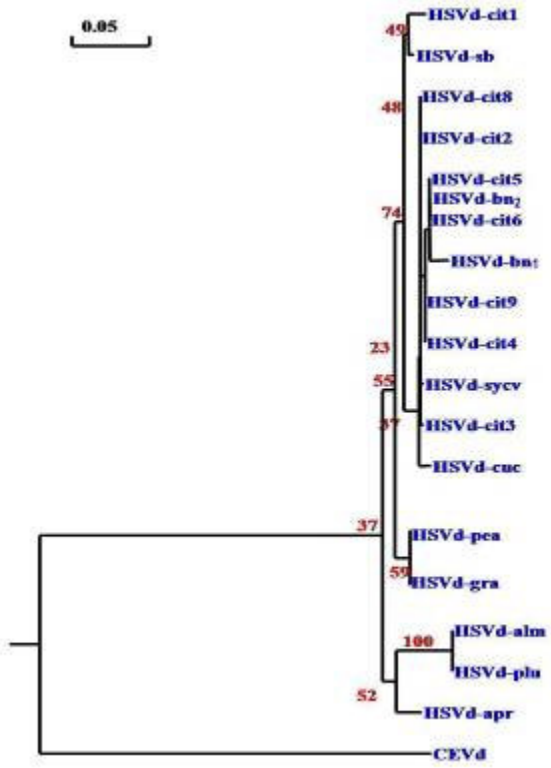

Figure 4. A phylogram drawn through neighbor joining bootstrap method in CLUSTAL X (1.81b) software, illustrating phylogenetic position of HSVd-bn1and HSVd-bn-2 among the HSVd variants. See Table 2 for a specification of viroid variants.

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% identitical 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

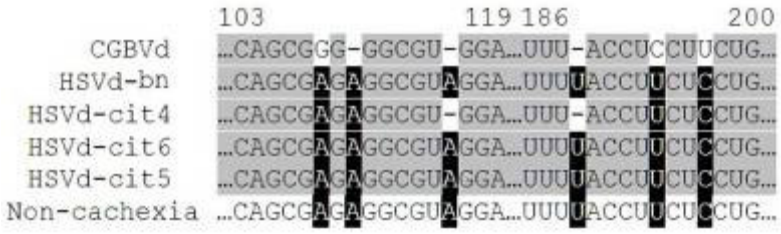

Figure 5. A comparison of the cachexia expression motif of HSVd-bn (-1 and -2) and some other citrus variants of HSVd. Nucleotides: Non homologous sequences; Nucleotides: Conserved sequences; Nucleotides: Homologous sequences, (-): Lack of nucleotide.

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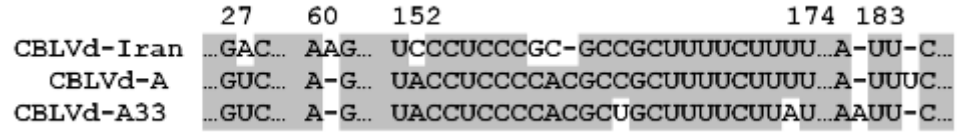

Figure 6. Comparison among primary sequence of CBLVd from Iran and two other highly similar CBLVd variants. Sequences are aligned for maximum homology using Vector NTI program (version 9.0.0). Nucleotides: Homologous sequences; (-): Lack of nucleotide; Nucleotides: Non homologous sequences.

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.

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[DOR: 20.1001.1.16807073.2014.16.3.18.6]

ترادف نوکلئوتیدی و ویژگی های ساختاری واریانت های ویروئید کوتولگی رازک و ویروئید پیچیدگی برگ مرکبات از استان کهگیلویه و بویراحمد

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چکیده

جدایه¬های ویروئید کوتولگی رازک (HSVd) به عنوان عامل کاککسیای مرکبات در مازندران معرفی شده¬اند و اخیراً مشخص گردیده است که این ویروئید با بیماری زرد و چوب پنبه¬ای شدن رگبرگ پرتقال و عارضه شقاقی لیموشیرین در استان فارس در ارتباط است. در این مطالعه ویروئیدهای همراه با درختان مرکبات دارای سوختگی سرشاخهٔ صمغ¬دار در استان کهگیلویه و بویراحمد جداسازی و برخی ویژگی¬های آن¬ها گزارش گردیده است. درختان پرتقال و لیموشیرین پیوند شده روی نارنج که دچار نکروز پوست و ترشح صمغ از تنه و ساقه و سرخشکیدگی بودند از منطقه دهدشت نمونه برداری شده و به کمکRT-PCR ، همسانه¬سازی و تعیین ترادف مورد بررسی قرار گرفتند. نمونه¬ها به روش الیزا و جهت ردیابی ویروس تریستزا نیز بررسی شدند. دو واریانت HSVd و دو واریانت ویروئید پیچیدگی برگ مرکبات که با ترادف¬های بانک ژن متفاوت بودند در تمام نمونه¬های دارای علایم شناسایی گردید. یک واریانت ویروئید اگزوکرتیس مرکبات نیز فقط در لیموشیرین های دارای علایم یافت شد. یکی از جدایه¬های HSVd (HSVd-bn1) برای مقایسه با تعدادی از واریانت¬های استان¬های فارس، مازندران و دیگر ترادف¬های بانک ژن مورد استفاده قرار گرفت. بر اساس ترادف نوکلئوتیدی و آنالیزهای ساختار ثانویه، HSVd-bn1و HSVd-bn2 جزء واریانت¬های غیر کاککسیای این ویروئید قرار گرفته و ۹۵ درصد تشابه با ویروئید ترشح صمغ تنه را نشان دادند. از سوی دیگر، ویروس تریستزا در گیاهان آلوده ردیابی نشد. هنوز بایستی مشخص شود که نکروز تنه لیموشیرین و پرتقال بوسیلهٔ ویروئید(ها) ایجاد می¬شود و یا سایر عوامل نیز دخالت دارند

[DOR: 20.1001.1.16807073.2014.16.3.18.6]