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Molecular Variability of *Hop stunt viroid:* Identification of a Unique Variant with a Tandem 15-nucleotide Repeat from Naturally Infected Plum Tree

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Abstract For this study, 68 plum samples were collected from 12 provinces of China. Low molecular weight RNAs were extracted and used for dot-blot, reverse transcription–polymerase chain reaction (RT-PCR), return–polyacrylamide gel electrophoresis, and biological indexing using cucumber. Results showed that 15 out of the 68 plum samples were positive for *Hop stunt viroid* (HSVd). Four positive samples were selected for cloning and sequence analysis. Results indicated that most HSVd sequences from the plum in China had 1–3 nucleotide changes from the closest HSVd in GenBank. In addition, the sample PB21 (cv. 'Friar') collected from Hebei province had one sequence with a 15-nt duplication (named HSVd D-15) at the 244/245 position in the lower central region. By biological indexing, cucumber seedlings, an indicator plant for HSVd, were inoculated with RNAs directly extracted from the original plum source (PB21, cv. 'Friar'). Nucleotide sequencing analysis of the progeny showed that HSVd, but not HSVd-D15, was recovered from inoculated cucumbers. Although very unlikely, the possibility that this extra sequence was the result of a PCR artifact cannot be completely ruled out.

Keywords *Hop stunt viroid* · Plum · Recombination · Intramolecular recombination · Sequence repeat

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Introduction

Viroids are the smallest known plant pathogens, consisting of a noncoding, single-stranded, 246–475 nt circular RNA that is autonomously replicated by enlisting host-encoded proteins (Flores et al. 1997), mediated through specific conformations (Owens et al. 1986; Visvader et al. 1985). *Hop stunt viroid* (HSVd) belongs to the Pospiviroidae family and is found in a wide range of hosts, including hop, cucumber, grapevine, citrus, plum, peach, pear (Shikata 1990), apricot, and almond (Astruc et al. 1996; Cañizares et al. 1999).

Genome enlargement has been reported in only two known viroids, *Coconut cadang–cadang viroid* (CCCVd), and *Citrus exocortis viroid* (CEVd). A series of terminal repeats containing 41, 50, 55, and 100 nucleotides were detected that were unique to CCCVd (Haseloff et al. 1982). Unusual variants of CEVd D-92, D-104 from a hybrid tomato (*Lycopersicon esculentum* Mill. × *Lycopersicon peruvianum*) (Semancik et al. 1994; Semancik and Duran-Vila 1999) and CEVd D-96 from eggplant (Fadda et al. 2003), have been reported. Recently, the transmission of CEVd D-92 and D-104 to *Gynura aurantiaca* demonstrated that other plant species were also capable of processing and replicating these structures. Two additional enlarged CEVd variants (D-87 and D-76) and three transient forms (D-38, D-40, and D-43) from hybrid tomato have been reported recently (Szychowski et al. 2005).

This report describes the molecular variability of *hop stunt viroid* (HSVd) present in plum trees of different regions in China and an unusual variant of HSVd, temporarily named HSVd D-15, which originated from a natural host plum.

Materials and Methods

Plant and Viroid Sources

The 68 plum samples were collected from 12 provinces of China. The samples consisted of six leaf samples (Nos. PL1-PL6) and 62 bark samples (Nos. PB1-PB62) (Table 1).

Preparation of Low Molecular Weight RNAs

Low molecular weight RNAs were extracted according to Li et al. (1995). In brief, 2 g of tissue was powdered in liquid nitrogen, extracted with 4 ml of 1M K_2HPO_4 containing 0.1% 2-mercaptoethanol, and homogenized with 4 ml phenol:chloroform (1:1, v/v). After eliminating polysaccharides by 2-methoxyethanol extraction and CTAB precipitation, 2M LiCl was used to precipitate low molecular weight RNAs. The resulting preparation was dissolved in 20 µl distilled water.

Dot-blot Hybridization

RNA extracts of 2 µl and diluted plasmid controls were heated for 15 min at 65°C, quickly chilled on ice, and applied to nylon membranes (Hybond-N^+ Amersham

Table 1 Sources of plum samples

Collection location (City or Province)	Number of samples	Sample number and cultivar[a]
Beijing	6	PL1 Dahongmeigui; PL2 Friar; PL3 Qiuji; PL4 Lanbaoshi; PL5 Dashizaosheng; PL6 Ruby
Inner Mongolia	14	PB1-PB14 Dazili
Hubei	1	PB15 Unknown
Hebei	6	PB16 Lanbaoshi; PB17 Tianlizi; PB18 Suanlizi; PB19 Unknown; PB20 Ruby; PB21 Friar
Shandong	2	PB22-PB23 Huanglizi
Heilongjiang	2	PB24 Mudanjiang 1; PB25 Mudanjiang 3
Shaanxi	12	PB26 Ribenzaohong; PB27 Australia 14; PB28 Benmei; PB29 Banunu; PB30 Ruby; PB31 Qiuji; PB32 Australia 14; PB33 Angeleno; PB34 Lümei; PB35 Queen gold; PB36-37 Benli
Guangxi	1	PB38 Unknown
Xinjiang	3	PB39-41 Huanglizi
Shanxi	10	PB42 Suilenghong; PB43 Misili; PB44 Dahongli; PB45 Aodeluoda; PB46 Xianfeng; PB47 Hongrouli; PB48 Queen rose; PB49 Friar; PB50 LI 6; PB51 Dashizaosheng
Jilin	10	PB52 Jilin 1; PB53 Wanhong; PB54-55 Tianlizi; PB56-57 Huanglizi; PB58 Wanhong; PB59 Jilin 1; PB60 Huanglizi; PB61 Dandongzajiao 1
Fujian	1	PB62 Sutaixianli

[a] PL, plum leaf. PB, plum bark

Biosciences). These were hybridized overnight at 50°C using a HSVd-specific DNA probe labeled with digoxigenin using the DIG High Prime DNA Labeling and Detection Starter Kit 1 (Roche).

RT-PCR Amplification, Cloning, and Sequencing

In order to detect and clone viroids from the plum and inoculated cucumber plants, RT-PCR primers were designed according to the HSVd sequence from the plum, based on acc. no. D13764 (Table 2). The primers lie in the strictly central conserved region (CCR) of HSVd and contain the unique endonuclease restriction site *Sma*I (underlined in Table 2). Reverse transcription was conducted using 3 µl RNA extract, 4 µl M-MLV 5 × buffer, 4 µl 2.5 mmol/l dNTPs, 2 U/µl RNasin, 1 µl R1 primer (20 µmol/l), and 10 U/µl M-MLV reverse transcriptase. Water was added to a final volume of 20 µl. The cDNA synthesis was conducted at room temperature for 10 min and 42°C for 1 h.

The PCR reaction utilized 25 µl 2 × *Taq* PCR MasterMix, 1 µl of each primer pair (R2 and F3; 20 µmol/l), and 3 µl first-strand cDNA reaction mixture. Water was added to a final volume of 50 µl. PCR parameters consisted of 94°C for 5 min and 30 cycles of 94°C for 30 s, 53°C for 30 s, and 72°C for 30 s, with a final extension step of 72°C for 7 min. After RT-PCR, electrophoresis confirmed the

Table 2 Primer sequence of HSVd for RT-PCR

Primers	Polarity	Sequence[a]	Position	RE
RT- R1	–	5′-GCTGGATTCTGAGAAGAGTT-3′	106-87	
PCR- R2	–	5′-AACCCGGGGCTCCTTTCTCA-3′	84-67	*Sma*I
PCR- F3	+	5′-AACCCGGGGCAACTCTTCTC-3′	79-96	*Sma*I

[a] Underlining indicates the unique endonuclease restriction site *Sma*I

presence of a PCR product of the expected size. The products were purified with the PCR purification kit (Tiangen). The resulting fragments were cloned into a pGEM-T vector (Promega) and transformed into *E. coli* DH5α. The cDNA clones from all isolates were identified by restriction analysis. Selected clones were sequenced using an automated DNA sequencer (ABI Prism 3730XL DNA Analyzer) and analyzed by DNAman Version 5.2.2.

R-PAGE

RNA extracts were separated by return–polyacrylamide gel electrophoresis (R-PAGE) under nondenaturing and denaturing conditions, and revealed by silver staining (Li et al. 1995).

Biological Indexing

Cucumber (*Cucumis sativus* L. Suyo) seedlings of the cotyledon stage were inoculated with low molecular weight RNA extracted from plum, dissolved at various concentrations in 100 mmol/l Tris–HCl; pH 7.5, 10 mmol/l EDTA.

Prediction of the Secondary Structure of Viroid RNA

The predicted secondary structures of minimum free energy for the viroid RNAs were analyzed using an Mfold RNA folding package available on the Internet (Mfold version 3.0, http://www.bioinfo.rpi.edu/applications/mfold/old/rna/form3.cgi, or version 0.01, http://www.bibiserv.techfak.uni-bielefeld.de/mfold/).

Results

Dot-blot Hybridization

Dot-blot hybridization revealed that 15 of the 68 plum samples were positive for HSVd (Fig. 1). The positives included one leaf sample (PL5) and 14 bark samples (PB2, 6, 14, 16, 18, 19, 21, 45, 48, 53, 55, 56, 57, 61), collected from Beijing, Inner Mongolia, Hebei, Xinjiang, and Shanxi provinces in China.

Fig. 1 Dot-blot hybridization with a DIG-labeled cDNA probe against HSVd in the plum. Circled samples are those selected for RT-PCR, cloning, and sequence analysis. NC is the healthy control and PC is the pGEM-T-HSVd cDNA control

Cloning and Sequence Analysis

Four positive samples were selected for cloning and sequence analysis. Five independent cDNA clones were sequenced for each sample (Table 3). After comparing the sequence obtained from this isolate with the previously reported HSVd in GenBank, we found that most HSVd sequences from the plum in China had 1–3 nucleotide changes from the HSVd sequence in GenBank. However, in one sample PB21 (cv. 'Friar') collected from Hebei province, a sequence with a 15 nt duplication (named HSVd D-15) at the 244/245 position was found in the lower part of the central region. More clones from this sample were selected for sequence analysis. Thirteen individual clones were sequenced; in addition to eight normal HSVd clones, five enlarged HSVd D-15 clones were obtained. The five D-15 clones all displayed a 15 nt duplication at the 244/245 position (Fig. 2). The result of *Sma*I restriction enzyme analysis showed that a unit length cDNA fragment of HSVd D-15 migrated slower than normal HSVd unit length cDNA (Fig. 3). Multiple alignment analysis of the two different sequences of HSVd D-15 (A and B) with the sequence of HSVd from the sample PB21 (acc. no. EF076831) is shown in Fig. 4.

R-PAGE Analysis

In order to detect the HSVd circular RNA structure, RNA extracts were obtained from the plum samples PB6, PB16, PB19, and PB21 and electrophoresed in 5%

Table 3 Analysis of HSVd sequence variants obtained using the HSVd in GenBank

Province	Variety	Number of clones	Accession No.	Size (nt)	Closest HSVd variant	Nucleotide difference from closest sequence
Beijing	Dashizaosheng	5	DQ648600	297	Y09345[a]	$G^{107} \rightarrow A$
			DQ648601	297	Y09345	$C^{59} \rightarrow T$, $G^{107} \rightarrow A$
Inner Mongolia	Unknown	5	EF076822	297	AY425171[b]	$G^{111} \rightarrow T$
			EF076823	297	AY425171	$G^{107} \rightarrow A$, $G^{114} \rightarrow A$
			EF076824	297	Y09345	$G^{107} \rightarrow A$
			EF076825	297	AY425171	$G^{49} \rightarrow A$, $G^{107} \rightarrow A$, $T^{238} \rightarrow C$
Hebei	Lanbaoshi	5	EF076826	298	Y09345	$-^{46} \rightarrow A$, $G^{107} \rightarrow A$, $T^{135} \rightarrow C$
			EF076827	297	Y09345	$A^{110} \rightarrow G$
			EF076828	297	AY425171	$G^{107} \rightarrow A$
			EF076829	298	Y09345	$-^{102} \rightarrow T$, $G^{107} \rightarrow A$
			EF076830	297	Y09345	$T^{18} \rightarrow C$, $G^{107} \rightarrow A$, $A^{160} \rightarrow G$
Hebei	Friar	8	EF076831	297	Y09345	–
			EF076832	297	Y09345	$G^{107} \rightarrow A$, $A^{118} \rightarrow C$, $A^{209} \rightarrow G$
			EF076833	297	Y09345	$G^{107} \rightarrow A$, $T^{171} \rightarrow C$,
			EF076834	297	AJ297830[c]	–
			EF076835	297	Y09345	$G^{107} \rightarrow A$, $T^{237} \rightarrow C$,
		5	NOT YET	312		15 nt at 244/245 position
			NOT YET	312		15 nt at 244/245 position

[a] Kofalvi et al. 1997; GenBank acc. no. Y09345 is the sequence of HSVd from apricot

[b] Lee et al., 2003; GenBank acc. no. AY425171 is the sequence of HSVd from plum

[c] Amari et al. 2001; GenBank acc. no. AJ297830 is the sequence of HSVd from apricot

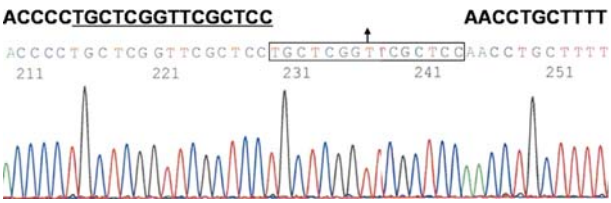

Fig. 2 Sequencing data of HSVd D-15 identified in the plum 'Friar'. The normal sequence is shown across the top in bold black letters. The underlined sequence shows the 15 nt repeat. The HSVd D-15 sequence is below the normal sequence. Boxed portion indicates a 15 nt duplication at the position between 244 and 245 in the normal

polyacrylamide gel using R-PAGE protocol. The results indicated that only one band corresponding to the normal class of circular HSVd molecule was detectable in all the samples, including PB21 (cv. 'Friar'), from which HSVd D-15 was successfully

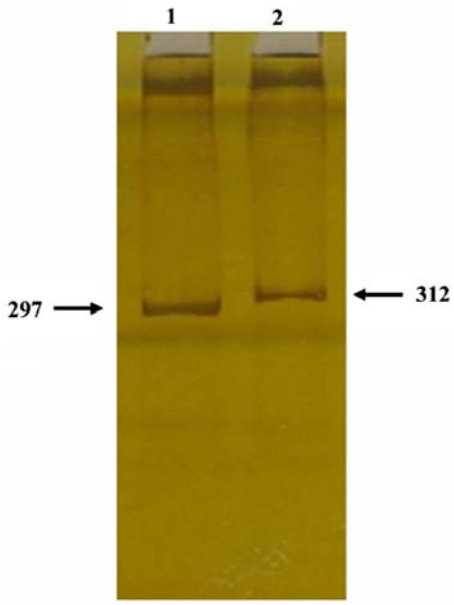

Fig. 3 *Sma*I restriction digestion analysis of recombinant plasmid DNA containing HSVd cDNA. The recombinant plasmid DNA was digested with *Sma*I, electrophoresed in 8% PAGE, and stained with silver. Lane 1, pGEM-T-HSVd. Lane 2, pGEM-T-HSVd D-15

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EF076831    CTGGGGAATTCTCGAGTTGCCGCAAAAGGCATGCAAAGAAAAAAACTAGGCAGGG     55
HSVdD-15A   CTGGGGAATTCTCGAGTTGCCGCAAAAGGCATGCAAAGAAAAAAACTAGGCAGGG     55
HSVdD-15B   CTGGGGAATTCTCGAGTTGCCGCAAAGGGCATGCAAAGAAAAAAACTAGGCAGGG     55
Consensus   ctggggaattctcgagttgccgcaaa ggcatgcaaagaaaaaaactaggcaggg

EF076831    AGGCGCTTACCTGAGAAAGGAGCCCCGGGGCAACTCTTCTCAGAATCCAGCGAGA    110
HSVdD-15A   AGGCGCTTACCTGAGAAAGGAGCCCCGGGGCAACTCTTCTCAGAATCCAGCAAGA    110
HSVdD-15B   AGGCGCTTACCTGAGAAAGGAGCCCCGGGGCAACTCTTCTCAGAATCCAGCAAGA    110
Consensus   aggcgcttacctgagaaaggagccccggggcaactcttctcagaatccagc aga

EF076831    GGCGTGGAGAGAGGGCCGCGGTGCTCTGGAGTAGAGGCTCTGCCTTCGAAACACC    165
HSVdD-15A   GGCGTGGAGAGAGGGCCGCGGTGCTCTGGAGTAGAGGCTCTGCCTTCGAAACACC    165
HSVdD-15B   GGCGTGGAGAGAGGGCCGCGGTGCTCTGGAGTAGAGGCTCTGCCTTCGAAACACC    165
Consensus   ggcgtggagagagggccgcggtgctctggagtagaggctctgccttcgaaacacc

EF076831    ATCGATCGTCCCTTCTTCTTTACCTTCTTCTGGCTCTTCTTGGAGACGCGACCGG    220
HSVdD-15A   ATCGATCGTCCCTTCTTCTTTACCTTCTTCTGGCTCTTCTTGGAGACGCGACCGG    220
HSVdD-15B   ATCGATCGTCCCTTCTTCTTTACCTTCTTCTGGCTCTTCTTGGAGACGCGACCGG    220
Consensus   atcgatcgtcccttcttctttaccttcttctggctcttcttggagacgcgaccgg

EF076831    TGGCACCCCTGCTCGGTTCGCTCC..............AACCTGCTTTTGTTCT    260
HSVdD-15A   TGGCACCCCTGCTCGGTTCGCTCCTGCTCGGTTCGCTCCAACCTGCTTTTGTTCT    275
HSVdD-15B   TGGCACCCCTGCTCGGTTCGCTCCTGCTCGGTTCGCTCCAACCTGCTTTTGTTCT    275
Consensus   tggcacccctgctcggttcgctcc               aacctgcttttgttct

EF076831    ATCTGCGCCTCTGCCGCGGATCCTCTCTTGAGCCCCT                      297
HSVdD-15A   ATCTGCGCCTCTACCGCGGATCCTCTCTTGAGCCCCT                      312
HSVdD-15B   ATCTGCGCCTCTACCGCGGATCCTCTCTTGAGCCCCT                      312
Consensus   atctgcgcctct ccgcggatcctctcttgagcccct
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Fig. 4 Multiple alignment analysis of two types of HSVd D-15 (A and B) with the normal HSVd. Shaded portions indicate a 15 nt duplication at the position between 244 and 245 in the normal sequence

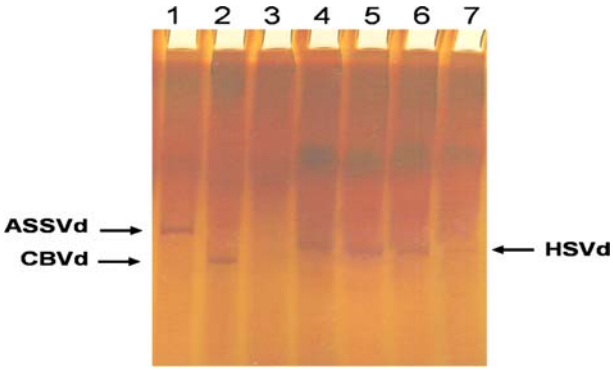

Fig. 5 Detection of the HSVd circular RNA structure directly from plums by R-PAGE and silver staining. Lane 1, Apple scar skin viroid (ASSVd). Lane 2, *Coleus blumei* viroid (CBVd). Lane 3, healthy plum control. Lane 4, plum PB6. Lane 5, plum PB16. Lane 6, plum PB19. Lane 7, plum PB21 (cv. 'Friar'). HSVd D-15 was amplified from the sample PB21, but no additional band was detectable

amplified (Fig. 5). It is likely that the concentration or the replication of HSVd D-15 in the original plum tissue was too low to detect it directly by R-PAGE analysis.

Biological Indexing

In order to investigate their biological properties further, cucumber seedlings were inoculated with RNA directly extracted from the original plum source (PL5, PB6, PB16, PB21). Approximately six weeks post inoculation, the cucumber showed obvious symptoms; i.e., the leaf blades became small and undulated, and their edges turned downward. Internodes from the younger parts of infected plants were shorter than those of healthy plants, and the whole plant had become stunted. Symptoms of the cucumbers inoculated with RNA directly extracted from PB21 were similar to those of PL5, PB6, and PB16. Then the low molecular weight RNA was extracted from symptomatic leaves of cucumbers inoculated with PB21 and analyzed for the presence of HSVd D-15 using dot-blot, RT-PCR, cloning, and sequencing. We have screened 50 independent HSVd cDNA clones by *Sma*I digestion assay, but only HSVd of normal size was detectable. We have also sequenced four cDNA clones from the progeny propagated in cucumber. The sequences are compared with those in the original source (i.e., plum cv. 'Friar') in Table 4.

Table 4 Comparison of the progeny sequences of HSVd propagated in cucumber and in the original plum (cv. 'Friar')

Host	Clone	Number of Clones	Size (nt)	Closest HSVd Variant	Nucleotide Difference from Closest Sequence
Cucumber	C1	2	297	EF076831	$C^{59} \rightarrow A$, $G^{60} \rightarrow A$, $G^{107} \rightarrow A$
	C2	1	297	EF076831	$C^{59} \rightarrow A$, $G^{107} \rightarrow C$, $T^{190} \rightarrow C$
	C3	1	297	EF076831	$C^{59} \rightarrow T$, $C^{102} \rightarrow A$, $A^{209} \rightarrow G$

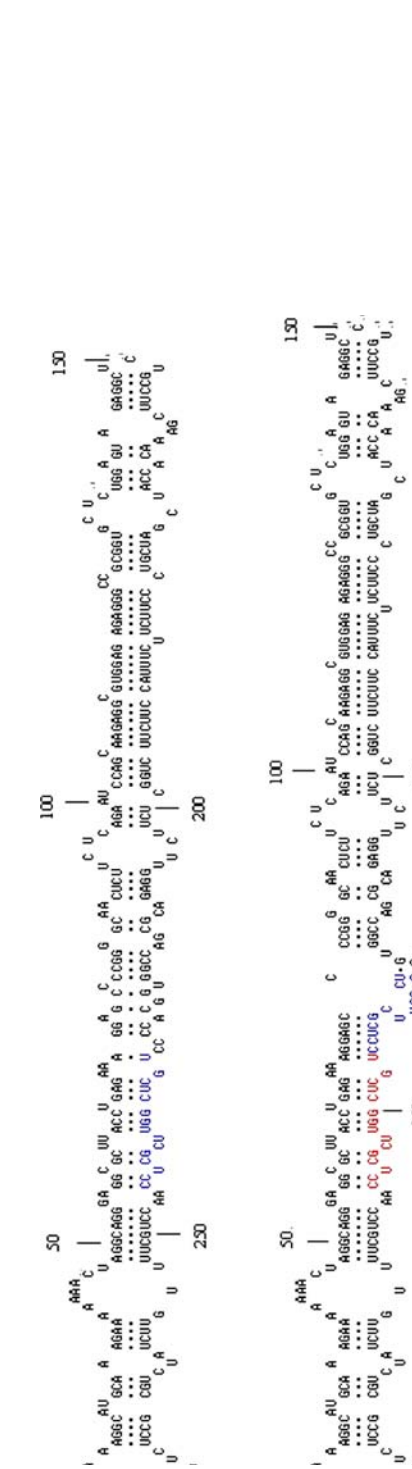

Fig. 6 Predicted rodlike secondary structures (Mfold version 3.0) of the normal HSVd (**A**) and HSVd D-15 (**B**). The blue and red sequences show the 15 nt repeat. Note that the predicted overall secondary structure is stably maintained in HSVd D-15

Nucleotide Sequences and Secondary Structure of HSVd and HSVd D-15

The predicted secondary structure of minimum free energy was the highly base-paired rodlike structural characteristic of HSVd and HSVd D-15 (Fig. 6). The overall base pairing of the molecule seemed to be stably maintained in HSVd D-15, except for the region corresponding to the duplicated sequence.

Discussion

The results of cloning and sequence analysis showed a certain degree of sequence variation for plum isolates of HSVd in China. Most HSVd sequences from the plum had 1–3 nucleotide changes, as compared with the closest HSVd in GenBank; however, they were not differentiated from the other stone fruit isolates reported in other parts of the world (Sano et al. 1989; Kofalvi et al. 1997; Amari et al. 2001). In fact, the sequence of HSVd (acc. nos. EF076831 and EF076834) from plum (cv. 'Friar') was identical with the one from apricot (acc. no. Y09345 and AJ297830) in Spain and Turkey, respectively. All results suggest that there is no clear relationship among the type of sequence variation, host specificity, and geographic origin in HSVd plum isolates.

The exact process of HSVd D-15 generation is unknown. Genome enlargement observed in CEVd and CCCVd has helped to identify the mechanisms responsible for genome enlargement in viroids. Since HSVd D-15 was not stably maintained in cucumber, it would be suggested that this is a poor host for HSVd D-15 or that HSVd D-15 could not effectively compete with HSVd in the setting of a mixed infection. Moreover, "elongated" forms of CEVd were reported in plants that had been inoculated for an extensive period of time. The other possibility is that two months of cucumber infection could not sustain or build up a high titer of HSVd D-15. It is well accepted, however, that a similar event takes place on HSVd from fruit trees during long incubation periods and that long incubation under cultivation produces variable mutations in viruses and viroid populations. Although very unlikely, the possibility that this extra sequence was the result of a PCR artifact cannot be completely ruled out.

To our knowledge, this is the first report of HSVd duplication. Further investigation is required to obtain purified HSVd D-15 from the plum (cv.'Friar') and to examine its biological properties.

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