Multiple Citrus Viroids in Citrus from Japan and Their Ability to Produce Exocortis-Like Symptoms in Citron

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ABSTRACT

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Sequential polyacrylamide gel electrophoresis analyses showed many viroid-like RNAs in samples collected from citrus trees in Japan. Reverse transcription polymerase chain reaction and sequencing analyses of the amplified fragments verified that they were derived from variants of six citrus viroids, *Citrus exocortis viroid* (CEVd), *Citrus bent leaf viroid* (CBLVd) including CVd-I-LSS (a distinct variant of CBLVd), *Hop stunt viroid*, *Citrus viroid III*, *Citrus viroid IV*, and *Citrus viroid OS*. The

samples induced symptoms with variable severity in Arizona 861-S1 'Etrog' citrons (*Citrus medica* L.) likely due to the varying accumulation patterns produced by the different viroids. Some of the symptoms caused by the samples harboring the citrus viroids other than CEVd were as severe as those caused by CEVd. Some source citrus trees showing the severe bark scaling characteristic of exocortis disease in trifoliate orange (*Poncirus trifoliata* (L.) Raf.) rootstocks contained only citrus viroids other than CEVd in complex. This indicates that certain exocortis-like diseases in Japan were caused by some combination of citrus viroids not including CEVd.

Additional keywords: cachexia, nucleotide sequence, variation.

Viroids are the smallest known plant pathogens. They consist of unencapsidated, circular, and single-stranded RNA with 246 to 463 nucleotides (nt). Many citrus viroid variants have been catalogued using sequential polyacrylamide gel electrophoresis (sPAGE) (5). Nucleotide sequencing has been utilized to classify them (1,3,7,9–12,14,16–19,21,24,25,26) into six citrus viroids, *Citrus exocortis viroid* (CEVd), *Citrus bent leaf viroid* (CBLVd; formerly called citrus viroid [CVd]-I) including CVd-I-LSS (low sequence similarity: a distinct variant of CBLVd, or a possible new species [11]), *Hop stunt viroid* (HSVd; formerly called CVd-II), *Citrus viroid III* (CVd-III), *Citrus viroid IV* (CVd-IV), and *Citrus viroid OS* (CVd-OS). CBLVd including CVd-I-LSS, CVd-III, and CVd-OS are classified in the genus *Apscaviroid*, while CEVd, HSVd, and CVd-IV are classified in the genera *Pospi-viroid*, *Hostuviroid*, and *Cocadviroid*, respectively (7,11,12).

Some citrus viroids affect citrus trees grafted on trifoliate orange (*Poncirus trifoliata* (L.) Raf.) rootstocks (2,6,8,22,25). In particular, CEVd is the causal agent of citrus exocortis disease, the symptoms of which are bark scaling on trifoliate orange rootstocks and stunting of the entire tree (6). On the other hand, some HSVd variants are causal agents of citrus cachexia disease, which have not been found in Japan, and can severely affect sensitive hosts such as mandarins (*Citrus reticulata* Blanco) (6,14,20,21). However, whereas citrus viroids can be influential in citriculture in Japan where trifoliate oranges and mandarins are the most

popular rootstocks and cultivars, respectively, the distribution of citrus viroids in Japan has been only fragmentarily investigated (9,11,12,15,16,24,27). Therefore, in this study, we examined the presence of these viroids in citrus trees within Japan by means of sPAGE, reverse transcription polymerase chain reaction (RT-PCR), and sequencing. We determined that multiple citrus viroids were present in single trees and compared the symptoms produced by these infections to those produced by exocortis disease in Japan.

MATERIALS AND METHODS

Plant samples. Plant samples used in the present study were collected from diseased, declining, or stunting citrus trees planted in certain horticultural experimental stations or commercial orchards in every representative citrus growing area in Japan (Table 1). Severe bark scaling characteristic of exocortis disease was observed in the trifoliate orange rootstocks of some of the source citrus trees during sample collection. All the samples were graft-inoculated into Arizona 861-S1 'Etrog' citrons (*C. medica* L.) on rough lemon (*C. jambhiri* Lush.) rootstocks, and maintained in a greenhouse.

Extraction of nucleic acids and sPAGE analysis. Extraction of nucleic acids from samples for sPAGE was conducted as described by Ito et al. (11) in a fifth scale with 30 g of leaves and stems of the citrons as the primary extract sources. When the citron tissues were not sufficient for 30 g, extract of the suckers of the rough lemon rootstock budded with the citron was added. However, in this case, the same volumes described above sometimes made further purification difficult. Therefore, after phenol/

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chloroform extraction, approximately one-half volume of the supernatant was collected and diluted with one-half volume of extraction buffer.

Extracted nucleic acids were analyzed using an sPAGE system following the method described by Ito et al. (11) with slight modification. A second electrophoresis under the denaturing conditions of sPAGE was run for 3 h after the xylene cyanol tracking dye had left the gel. After the electrophoresis, the viroid-like RNAs were viewed by staining them with Silver Stain 'Daiichi' (Daiichi Pure Chemicals, Tokyo, Japan), following the manufacturer's instructions.

RT-PCR, and cloning and sequencing of the amplified fragments. RT-PCR to detect CEVd, CBLVd, CVd-I-LSS, CVd-

OS, HSVd, CVd-III, and CVd-IV was carried out following the method described by Ito et al. (11) using a primer pair designed to amplify the full length of each of the target viroids (Table 2). Thermal cycling was performed for 30 to 35 cycles. Amplified fragments from RT-PCR were viewed after electrophoreses in 6% polyacrylamide slab gel (8 × 10 × 0.1 cm) at 80 V for 1 h 45 min in TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.3) by silver staining as described above. The amplified fragments derived from variants whose nucleotide sequences were not determined (9,11,12,16) were cloned and sequenced primarily using the method described by Ito et al. (11). Some sequencing was conducted using *Taq* DNA polymerase and -21M13 dye primer or M13 reverse dye primer (Applied Biosystems, Foster

TABLE 1. Samples from source citrus trees used in this study, the viroid profiles, and the symptoms induced in Arizona 861-S1 'Etrog' citrons at 8 months postinoculation

Sample	Source (species)	Viroids detected by (sPAGE) and/or (RT-PCR) ^a							Symptoms ^b			
		CEVd	CBLVd	CVd-I-LSS	CVd-OS	HSVd	CVd-III	CVd-IV	E	L	B	S
AB	Allancio Belladonna (<i>Citrus sinensis</i> (L.) Osb.) ^c	D ^d	...	...	...	2 _d	D	...	NT ^e			
AD	Adamopoulos (<i>C. limon</i> (L.) Burm.) ^c	1 ^f	...	...	...	2 _{e,f}	2 _k	...	+++ ^g	++	+++	+++
BF	Bouquet de Fleurs (<i>C. aurantium</i> L.) ^c	1	...	...	...	3 _{d,f,g}	D	...	NT			
E127	Tachibana (<i>C. tachibana</i> (Mak.) Tan.)	1	...	...	...	1 _h	D	...	NT			
E55K ^h	Oochou Villafranca (<i>C. limon</i>) ^c	1 _a	...	...	...	1	D	...	NT			
FA	Feminello Apireno (<i>C. limon</i>) ^c	D	...	...	...	1	D	...	NT			
FO	Feminello Ovale (<i>C. limon</i>) ^c	1	...	...	...	2 _f	D	...	NT			
KA	Karystini (<i>C. limon</i>) ^c	1	...	...	...	...	D	...	NT			
MA	Maglene (<i>C. limon</i>) ^c	1	...	...	...	2 _f	1 _i	1	+++	+++	+++	+++
WS ⁱ	St. Michael orange-Wakayama (<i>C. sinensis</i>) ^c	1 _a	...	2	...	2 _i	3 _i	...	+++	++	+++	+++
E180 ⁱ	Clementine de Nules (<i>C. reticulata</i> Blanco)	...	1	...	...	1	1 _k	...	++	+++	++	++
E83AK ^{h,i}	Satsuma (<i>C. reticulata</i>) ^c	...	1	...	...	1 _h	2 _{m,n}	...	++	++	++	++
MC	Murcott (<i>C. reticulata</i> × <i>C. sinensis</i>)	...	1	...	...	1 _i	1 _n	1 _t	++	++	++	+++
TS ⁱ	Shiranui [(<i>C. reticulata</i> × <i>C. sinensis</i>) × <i>C. reticulata</i>] ^j	...	1	1	1 _b	1 _i	2	1 _t	++	+++	+++	++
9K-S ⁱ	Shiranui	...	...	1	1	1 _e	2 _k	...	++	++	++	++
MK	Kiyomi (<i>C. reticulata</i> × <i>C. sinensis</i>)	...	...	...	1	1	2 _{k,m}	...	+	++	+	++
OS ⁱ	Shiranui ^j	...	...	...	1 _b	1 _i	2 _{o,p}	...	+	++	++	++
10SA1-IW ⁱ	Ueno Wase (<i>C. reticulata</i>)	...	...	...	1 _c	2	2 _n	...	++	+++	++	++
11Y1-S ⁱ	Shiranui	...	...	...	1 _c	1 _e	1 _q	...	+	+++	++	+
3Y3-S ⁱ	Shiranui	...	...	...	1	2 _{e,g}	1 _q	...	+	++	++	++
OS+E120P8 ^k	Shiranui, and Tsutsumi navel (<i>C. sinensis</i>)	...	...	...	1 _b	2 _{i,j}	4 _{o,p,r,s}	...	++	+++	++	++
BC	Buckeye (<i>C. sinensis</i>) ^c	...	...	...	...	D	D	...	-	++	++	++
E120P8 ⁱ	Tsutsumi navel	...	...	...	...	1 _j	2 _{r,s}	...	+	+++	++	++
E146	Eureka (<i>C. limon</i>) ^c	...	...	...	...	D	D	...	+	++	++	+
ADG ^l	Adamopoulos ^c	1	...	...	...	...	...	...	++	++	++	+++
VF10-S ⁱ	Shiranui	...	...	1	...	...	...	...	+	+	++	+
OK	Tankan (<i>C. sinensis</i>)	...	...	...	...	1 _d	...	...	-	-	+	-
E130 ⁱ	Oomishima navel (<i>C. sinensis</i>)	...	...	...	...	...	1	...	-	++	++	+
LL	Lisbon (<i>C. limon</i>) ^c	...	...	...	...	...	1 _k	...	-	+++	++	+
LE	Lee (<i>C. reticulata</i>) ^c	...	...	...	...	...	...	1	-	++	++	++

^a (sPAGE) = sequential polyacrylamide gel electrophoresis. (RT-PCR) = reverse transcription-polymerase chain reaction. CEVd = *Citrus exocortis viroid*, CBLVd = *Citrus bent leaf viroid* formerly called citrus viroid (CVd)-I including CVd-I-LSS (low sequence similarity; a distinct variant of CBLVd, or a possible new species), CVd-OS = Citrus viroid OS, HSVd = *Hop stunt viroid*, CVd-III = *Citrus viroid III*, CVd-IV = *Citrus viroid IV*. CBLVd, CVd-I-LSS, and CVd-OS could not be clearly distinguished from one another using sPAGE because of their similar molecular sizes (11,12).

^b E = epinasty; L = leaf bending; B = midvein and petiole browning; S = stunting.

^c Citrus trees grown in fields in Department of Citrus Research, National Institute of Fruit Tree Science, Okitsu, Shizuoka.

^d D = detected by both of sPAGE and RT-PCR. ... = detected by neither sPAGE nor RT-PCR.

^e Not tested.

^f Numbers of variants whose nucleotide sequences were determined are indicated. Numbers less than those of viroid bands detected by sPAGE are underlined. Variants with the identical nucleotide sequences are indicated with the same letters (a-i) at the foot.

^g Symptom severity from the average of three plants after 8 months under controlled temperatures (28 to 32°C). - = no reaction to +++ = severe symptom.

^h Samples obtained by reciprocally cutting the source citrus tree and citron stems with a knife.

ⁱ Samples also described in previous reports (9,11,12,15,16).

^j Citrus trees grown in fields in Department of Citrus Research, National Institute of Fruit Tree Science, Kuchinotsu, Nagasaki.

^k Coinoculated with the OS and E120P8 samples.

^l Sample obtained by cutting a citron stem with a knife contaminated by cutting *Gynura aurantiaca* previously inoculated with the AD sample.

City, CA) employing a DNA sequencer (373A, Applied Biosystems). More than three cloned fragments were always sequenced to determine the nucleotide sequence of each variant. The determined sequences were analyzed using GENETYX Software version 10.1 (Software Development Co., Ltd., Tokyo, Japan).

Biological indexing. Each of the plant samples was graft-inoculated into three Arizona 861-S1 'Etrog' citrons on rough lemon rootstocks growing under controlled temperatures (28 to 32°C). Symptoms such as epinasty, leaf bending, midvein and petiole browning, and stunting shown in the citrons were evaluated 8 months after graft-inoculation (4).

RESULTS

sPAGE analysis, RT-PCR, and nucleotide sequencing analysis. sPAGE analyses of the nucleic acid extracts from the samples revealed various viroid-like RNAs (Table 1). To verify the presumed identities of the viroids from samples analyzed by sPAGE, RT-PCR was conducted on all samples to detect CEVd, CBLVd, CVd-I-LSS, CVd-OS, HSVd, CVd-III, and CVd-IV. Amplified fragments with molecular sizes specific for each were observed in silver-stained gels after electrophoreses (data not shown). Non-specific bands were seldom observed, and no fragments were detected from samples used as healthy controls (data not shown). The nucleotide sequencing analyses of most of the amplified fragments showed various variants derived from CEVd, CBLVd, CVd-I-LSS, CVd-OS, HSVd, CVd-III, and CVd-IV (Table 1). Although cloned fragments with nucleotide changes were sometimes obtained from some samples, the sequences were ignored unless three independent clones or more with identical sequences were obtained. Two variants or more were sometimes found in one sample, and some variants were identical to those in other samples. In most cases, the molecular sizes corresponded to those of the viroid-like RNAs detected using sPAGE (Table 1). The combination of CEVd, HSVd, and CVd-III was the most commonly detected combination from samples derived from the introduced cultivars such as lemons and oranges in the Department of Citrus Research, National Institute of Fruit Tree Science, Okitsu, Shizuoka (Table 1). On the other hand, the combination of CVd-OS, HSVd, and CVd-III was the most commonly detected combination from the other samples. Except for CEVd in the ADG sample and CVd-I-LSS in the VF10-S sample, both of which were artificially isolated, only HSVd, CVd-III, and CVd-IV were detected solely from the OK, E130, and LE samples, respectively.

Figure 1 shows the variation among the nucleotide sequences of the variants of CEVd, CBLVd including CVd-I-LSS, CVd-OS, HSVd, CVd-III, and CVd-IV. Variable nucleotide changes among the variants were dispersed in some of the structural domains. Some of the nucleotide sequences of the HSVd and CVd-III variants were identical to those of variants previously reported (10,19,21,25). The HSVd variant E55K had the same nucleotide sequence as CVd-IIC causing severe cachexia reaction (21). Also, the HSVd variants AB₂, AD₂, BF₃, FA, FO_{1,2}, MA₂, and WS₂ showed all or some of the six nucleotide changes associated with transition from noncachexia-inducing CVd-IIa to cachexia variants (20) (Fig. 1). All these HSVd variants were derived from source citrus trees such as lemons and oranges in the Department of Citrus Research, National Institute of Fruit Tree Science, Okitsu, Shizuoka (Table 1).

The nucleotide sequences of the CEVd variants AD, ADG, BF, E127, E55K, FO, KA, and MA, the CBLVd variants E83AK (11) and MC, the CVd-I-LSS variants TS, WS_{1,2}, and 9K-S (11), the CVd-OS variants MK, TS, 10SA1-IW, 3Y3-S, and 9K-S, the HSVd variants AB₂, AD_{1,2}, BF (1: omitted), E120P8, E127, E180, FA, FO (1: omitted), MA (1: omitted), MK, WS_{1,2}, and 10SA1-IW_{1,2}, the CVd-III variants AD_{1,2}, E120P8_{1,2}, E130, MA, OS_{1,2}, TS_{1,2}, WS_{1,2}, 10SA1-IW (2: omitted), and 11Y1-S, and the CVd-IV variants LE, MA, and MC (Fig. 1) have been deposited in GenBank (Accession Nos. AB054592-AB054641 and AB055634).

Biological indexing. Symptoms induced by each of the samples in the Arizona 861-S1 'Etrog' citrons at 8 months postinoculation are summarized in Table 1. The samples showed symptoms with variable severity in the citrons likely due to the varying accumulation patterns induced by different viroids. For example, coinoculation with the E120P8 and OS samples into the citrons (OS+E120P8) showed more severe symptoms than those expressed by the OS sample alone (Table 1). Consequently, some of the samples harboring citrus viroids other than CEVd in complex could cause symptoms as severe as those seen in the samples harboring CEVd.

Severe bark scaling in trifoliate orange rootstocks of some source citrus trees. Figure 2 shows examples of severe bark scaling associated with stunting of the entire tree observed in the trifoliate orange rootstocks of the source trees of the MC, TS, and E120P8 samples. No CEVd was detected from any of these samples (Table 1). Only HSVd and CVd-III were detected from all of them, whereas CBLVd and CVd-IV were additionally detected from the MC and TS samples, and CVd-I-LSS and CVd-OS was detected, as well, from the TS sample (Table 1). Trifoliate

TABLE 2. Primers used in the uniplex reverse transcription-polymerase chain reaction

Name	Sequence (5'-3')	Target ^a	Position	Reference ^b
CEV 20-mer primer (-) ^c	CCCTGAAGGACTTCTTCCCC	CEVd	71-90	Yang et al. (28)
CEV 24-mer primer (+)	ATCCCCGGGGAAACCTGGAGGAAG		91-114	
oC-CEVd (-)	CGGGGATCCCTGAAGGACTT	CEVd	77-96	Ben-Shaul et al. (3)
oS-CEVd (+)	GGGAAACCTGGAGGAAGTCG		97-116	
CBLV-CM (-)	ACGACCAGTCAGCTCCTCTG	CBLVd	75-94	Ito et al. (11)
CBLV-CP (+)	CGTCGACGAAGGCTCGTCAGCT		95-116	
CB2-CM (-)	ACGACCCTCAGTCTCCTCT	CVd-I-LSS	76-95	Ito et al. (11)
CB2-CP (+)	CGTCGACGAAGGCTCCTCAGTC		96-117	
CB3-CM (-)	ACGACAGGTGAGTTCTCCTT	CVd-OS	63-82	Ito et al. (12)
CB3-CP (+)	CGTCGACGAAGGCATGTGAGCT		83-104	
HSV 19-mer primer (-)	GGCTCCTTTCTCAGGTAAG	HSVd	60(61)-78(79)	Yang et al. (28)
HSV 25-mer primer (+)	CCGGGGCAACTCTTCTCAGAATCCA		79(80)-103(104)	
CVIII-CM (-)	TCCGACGACGACAGGTAAGTT	CVd-III	72-91	Rakowski et al. (19)
CVIII-CP (+)	CGAAGGCAGCTAAGTTGGTGA		92-112	
CVIV-CM (-)	GGATCCCTCTCAGGTATGT	CVd-IV	48-67	Puchta et al. (18)
CVIV-CP (+)	CCGGGGAAATCTCTTCAGACTC		68-89	

^a CEVd = *Citrus exocortis viroid*, CBLVd = *Citrus bent leaf viroid* formerly called citrus viroid (CVd)-I including CVd-I-LSS (low sequence similarity: a distinct variant of CBLVd, or a possible new species), CVd-OS = *Citrus viroid OS*, HSVd = *Hop stunt viroid*, CVd-III = *Citrus viroid III*, CVd-IV = *Citrus viroid IV*.

^b References to primers or viroid sequences used to design the primer.

^c (-) = antisense primer, (+) = sense primer.

CEVd	T1	P	V	T2	V	C	P	T1																		
	32	50	56	70	128	129	130	131	132	133	135	136	185	228	231	232	233	234	237	278	297	313	315	368		
<u>CEVc</u>	G	G	-	-	A	U	-	-	G	U	U	A	A	G	C	-	-	A	C	U	U	G	-	U	371 nt	
E127	A	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	C	-	U	C	-	U	371 nt (98.1%)	
E55K	A	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	C	-	U	C	-	U	371 nt (97.8%); WS	
BF	A	-	A	G	-	-	A	-	-	-	-	-	-	-	-	-	-	-	C	-	U	C	-	U	373 nt (97.3%)	
AD	A	-	-	-	C	-	-	A	A	-	-	-	-	-	U	U	G	-	-	C	-	U	C	-	U	371 nt (96.8%)
FO	A	-	A	G	-	-	A	G	-	-	-	-	-	-	-	-	G	-	-	C	-	U	C	-	U	372 nt (96.8%)
MA	A	A	A	G	-	-	A	-	-	-	-	-	-	-	-	-	-	-	A	C	-	U	C	-	U	373 nt (96.8%)
ADG	A	-	G	-	C	-	-	-	A	A	-	-	-	-	-	-	U	U	G	-	-	C	-	U	C	372 nt (96.5%)
KA	A	-	A	G	A	-	-	-	-	A	-	-	-	-	C	U	G	-	-	C	-	U	C	-	U	372 nt (95.5%)

CBLVd	T1	P	C	V	T2																																						
	28	37	48	49	50	51	55	62	63	64	68	73	74	83	87	88	110	115	117	119	123	124	125	127	128	129	138	147	148	150	151	152	153	154	151	152	153	155	157	159	160	162	168
<u>CBLVd</u>	A	A	-	-	-	A	U	-	-	A	G	G	-	-	U	G	C	-	G	-	U	U	-	-	G	-	C	G	-	-	-	-	-	G	U	U	-	C	C	U	C	U	
TS	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
MC	U	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
E180	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
E83AK	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
LSS (TS)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
LSS (VF10-S)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
LSS (9K-S)	U	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
LSS (WS ₁)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
LSS (WS ₂)	-	-	G	C	G	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	

(Cont.)	169	170	171	172	173	174	175	176	188	194	195	196	206	208	231	232	233	237	237	244	247	248	251	251	256	258	259	260	261	262	263	265	266	267	268	299	300			
<u>CBLVd</u>	A	A	-	-	-	-	-	-	G	C	-	-	-	-	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	318 nt	
TS	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	318 nt (98.1%)	
MC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	319 nt (96.6%)	
E180	C	U	U	C	U	U	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	328 nt (94.5%)
E83AK	C	U	U	C	U	U	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	328 nt (94.5%)
LSS (TS)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	328 nt (94.0%)	
LSS (VF10-S)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	327 nt (84.0%)	
LSS (9K-S)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	327 nt (83.1%)	
LSS (WS ₁)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	326 nt (82.8%)	
LSS (WS ₂)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	325 nt (82.7%)	
	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	330 nt (81.1%)	

CVd-OS	P	V	T2	V	C	P																							
	40	41	53	62	107	109	112	121	136	192	210	215	216	216	221	223	249	250	251	263	266	267	278	280	281	285	287		
<u>CVd-OS</u>	U	U	U	C	-	G	C	U	A	G	-	-	-	-	-	-	G	G	U	A	C	U	G	U	-	G	C	-	330 nt
9K-S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	329 nt (98.1%)	
MK	C	A	-	-	U	A	U	C	G	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	330 nt (96.1%)	
3Y3-S	C	A	A	U	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	329 nt (95.2%)	
10SA1-W	C	A	A	U	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	329 nt (94.5%); 11Y1-S	
TS	C	A	A	U	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	331 nt (94.3%); OS	

HSVd	T1	P	V	T2	V	C	P																							
	26	26	38	47	53	55	57	58	107	109	115	153	160	188	193	196	206	209	211	235	237	241	242	243	246	247	249	250	256	
<u>CVd-IIa</u>	-	G	A	-	G	G	A	G	A	A	A	A	A	U	U	C	-	G	G	-	C	U	C	G	U	U	C	A	-	302 nt
WS ₁	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	302 nt (98.7%); MC, TS, OS	
AD ₁	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	302 nt (98.3%); 9K-S, 11Y1-S, 3Y3-S	
MK	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	301 nt (98.3%)	
AB ₁	A	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	303 nt (98.0%); BF ₂ , OK, HSVd-citrus(T)	
BF ₁	U	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	303 nt (98.0%); 3Y3-S	
10SA1-W ₂	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	301 nt (98.0%)	
E120P ₂	A	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	303 nt (98.0%)	
10SA1-W ₁	A	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	302 nt (98.7%)	
E127	A	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	303 nt (98.3%); E83AK	
FO ₁	A	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	300 nt (98.3%)	
E180	A	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	301 nt (98.0%)	
MA ₁	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	299 nt (97.0%)	
FA	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	295 nt (95.0%)	
AD ₂	-	A	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	296 nt (93.7%); BF ₂ , FO ₂ , MA ₂	
AB ₂	A	A	G	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	297 nt (93.4%)	
E55K	-	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	295 nt (93.4%); CVd-IIc	
WS ₂	A	A	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	299 nt (92.1%)	

CVd-III	P	C	V	T2	V	C	P																															
	56	40	53	54	55	62	72	113	137	138	163	165	173	179	180	183	184	190	208	211	213	215	217	220	222	226	228	229	236	245	250	251	252	253	253	254	255	258
<u>CVd-IIa</u>	C	U	-	U	G	G	-	A	G	U	A	C	U	U	C	U	C	G	A	U	A	U	-	A	C	A	C	-	-	-	-</							

orange rootstocks of other graft-propagated trees of the source trees of the MC and TS samples commonly showed severe bark scaling (data not shown). On the other hand, the source tree of the E120P8 sample had not been graft-propagated and only this single tree showed severe bark scaling in its trifoliolate orange rootstock. Trifoliolate orange rootstocks of the source trees of the AD, MA, and WS samples harboring CEVd showed severe bark scaling as well (data not shown). The rootstocks of the other source trees were not checked, or bark scaling was not observed in them. Bark scaling was usually not observed in viroid-free trifoliolate orange rootstocks. Figure 2D shows an example of the absence of bark scaling in the trifoliolate orange rootstock of a viroid-free citrus tree whose tree age and environmental condition were similar with the source tree of the TS sample. Trifoliolate orange rootstocks from the viroid-free graft-propagated trees showed no bark scaling as well (data not shown). The MC, TS, and E120P8 samples were indexed by reciprocal stem-slash inoculation into *Gynura aurantiaca*, respectively, which did not result in symptoms associated with CEVd despite clear symptoms caused by all the samples harboring CEVd (data not shown).

DISCUSSION

The results of the RT-PCRs and subsequent sequence analysis of these products verified conclusions reached after sPAGE regarding the identities of citrus viroids in samples from citrus trees. Although a previous dot blot hybridization test showed positive signals of CEVd and CBLVd from the 3Y3-S sample (16), neither CEVd nor CBLVd were detected from the 3Y3-S sample (Table 1). Because another dot blot hybridization using a CEVd probe was also unable to detect CEVd from the 3Y3-S sample (data not

shown), the previous signals may have resulted from false positive reactions in dot blots. Nevertheless, all the results in this study showed that, in Japan, various citrus viroids have a complex distribution. CEVd, HSVd, and CVd-III in complex were most broadly detected from the introduced cultivars such as lemons and oranges in the Department of Citrus Research, National Institute of Fruit Tree Science, Okitsu, Shizuoka (Table 1). On the other hand, CVd-OS, HSVd, and CVd-III in complex were most broadly detected from the domestic cultivar, 'Shiranui' (Table 1). Some of the CEVd, CBLVd, HSVd, CVd-III, or CVd-IV variants had similar or identical nucleotide sequences to those in other countries (1,10,18,19,21,25,26) (Fig. 1), suggesting that those variants may have been imported from or exported to those countries together via citrus budwood (27). CVd-I-LSS and CVd-OS prevalent in Japan might be found in other countries as well. Surprisingly, the HSVd variants having similar or identical nucleotide sequences to cachexia-inducing variants (20,21) were identified (Fig. 1), whereas no cachexia disease has been reported in Japan. However, because all these HSVd variants were derived from nonsensitive citrus hosts (6) (Table 1), no typical cachexia symptoms were observed. Biological indexing using the indicator host, 'Parson's Special' mandarin (*C. reticulata*) (6,20,21), is now in progress to clarify whether cachexia disease is present in Japan.

Some nucleotide changes can affect the pathogenicity of a viroid, a phenomenon, which has also been observed in citrons infected with certain citrus viroid variants (14,17,25,26). Also, some combination of nucleotide changes in structural domains can be correlated with symptoms such as epinasty, stunting, and veinal necrosis (23). The citrus viroid variants found in this study had variable nucleotide changes dispersed in some of the structural domains (Fig. 1), which could reflect the variation of the symptom expressions in the citrons observed even among the samples having similar viroid profiles (Table 1). On the whole, symptoms expressing variable severity in the citrons were likely due to the varying accumulation patterns caused by the different viroids (Table 1), and some combination of citrus viroids other than CEVd induced symptoms as severe as those caused by CEVd. This finding indicates that citrus viroids other than CEVd in mixed infection produce severe exocortis-like symptoms in trifoliolate orange rootstocks. In fact, a combination of viroids with 330, 299, 295, and 284 nt that could be CBLVd, HSVd, CVd-III, and CVd-IV, respectively (2), produces severe bark scaling characteristic of exocortis disease in trifoliolate orange rootstocks (8). The analyses of the MC and TS samples harboring CBLVd, HSVd, CVd-III, and CVd-IV in common also showed that the combination could be associated with some exocortis-like diseases in Japan. On the other hand, the analyses of the E120P8 sample harboring only HSVd and CVd-III showed that only HSVd and CVd-III in complex could produce exocortis-like symptoms in trifoliolate orange rootstocks. However, another combination of HSVd and CVd-III with CBLVd in complex could not induce bark scaling but only bark striations and mild bark cracks in trifoliolate orange rootstocks (22). To clarify which viroid combination can produce exocortis-like symptoms, investigations using citrus trees inoculated with various mixtures of pure viroids grown in the same field should be performed. In any event, in Japan where trifoliolate orange rootstocks are commonly used and multiple citrus viroids can be broadly present in single trees, it is a matter of some concern that exocortis-like diseases could be caused by citrus viroids other than CEVd in complex.

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Fig. 2. Conditions of trifoliolate orange rootstocks of some of the source citrus trees and a viroid-free trifoliolate orange rootstock. **A**, Severe bark scaling in the rootstock of the 21-year-old 'Murcott' tree, the source of the MC sample harboring *Citrus bent leaf viroid* (CBLVd) formerly called citrus viroid (CVd)-I, *Hop stunt viroid* (HSVd), *Citrus viroid III* (CVd-III), and *Citrus viroid IV* (CVd-IV). **B**, Severe bark scaling in the rootstock of the 26-year-old orange tree at 8 years after top grafting of 'Shiranui' budwood, the source of the TS sample harboring CBLVd, CVd-I-LSS (low sequence similarity: a distinct variant of CBLVd, or a possible new species), Citrus viroid OS (CVd-OS), HSVd, CVd-III, and CVd-IV. **C**, Severe bark scaling in the rootstock of the 15-year-old 'Tsatsumi navel' tree, the source of the E120P8 sample harboring HSVd and CVd-III. **D**, No bark scaling in the rootstock of the 25-year-old Ponkan (*C. reticulata*) tree harboring no citrus viroids, which was grown in an adjoining section of the same field as the tree shown in B.

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