日本産セイヨウナシから分離されたナシblister cankerウイロイドについて

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Pear Blister Canker Viroid Isolated from European Pear in Japan*

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Abstract

A viroid was detected from European pears cultivated in Fukushima and Yamagata prefectures, Japan. Nucleotide sequence analysis of each of the Fukushima and Yamagata isolates revealed that they were isolates of pear blister canker viroid (PBCVd) reported in France. The Fukushima isolate consisted of 312 nucleotides, whereas the Yamagata isolate had 313 nucleotides. The only difference between the consensus sequence of the two isolates was an additional A residue in the Yamagata isolate at the site between no. 54 and 55 of the Fukushima isolate. The Fukushima isolate differed from the French isolate P2098T (Hernandez *et al.*, 1992) at 26 sites in the sequence, the Yamagata isolate at 27 sites. These changes included 17 base exchanges and six deletions for both isolates, with three insertions for the Fukushima isolate and four insertions for Yamagata. PBCVd was detected from six of twelve (50%) European rough bark (Sohi-byo in Japanese) pear samples in Fukushima prefecture and three out of eight (37.5%) dimple pit (Kubomika-byo in Japanese) samples in Yamagata, as opposed to none of nine European and eight Japanese symptomless pears.

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Key words: pear blister canker viroid, PBCVd, nucleotide sequence, rough bark, dimple pit.

INTRODUCTION

Etiological research in plant pathology does not seem to be as urgent these days, even though at least several diseases of perennials and orchard trees are believed to be caused by viruses or virus-like organisms which have not yet been identified. Since every pathogen is potentially dangerous, etiological research is very important, even if the damage incited by these diseases are not serious at the present. Both dimple pit (Kubomika-byo in Japanese) and rough bark (Sohi-byo in Japanese) on European pear trees reported in Yamagata and Fukushima prefectures in Japan[9-14] are diseases of unknown etiology. The diseases are now prevalent on cultivar La France, since its increased production in the last 10-15 years. Although the diseases were reported independently in Fukushima and Yamagata prefectures, they have some similarities such as in symptom expression and epidemiology[9,13,14]. For example, "Kubomika-byo", characterized by the dimple pit symptom on the fruit surface, is, in the most cases, accompanied with the rough bark symptom on their twigs[13,14]. Both diseases are graft-transmissible in pears[9-14], causing severe symptoms on cultivars La France, Marguerite Marillat, Silver Bell and Le Lectier, but none on cultivars Bartlett, Max Red Bartlett, Beurre Bosc or varieties of Japanese pears[9,13,14]. Epidemiological surveys revealed that the outbreaks of the two diseases were mainly associated with cultivar La France when they were top worked on contaminated Max Red Bartlett, which is a symptomless carrier of the diseases[9,13,14].

To investigate unknown causal agent of dimple pit and rough bark diseases on pear cultivating in Yamagata and Fukushima prefectures, we have done etiological examinations on the diseases and three viroids, that is PBCVd[3], ASSVd[8,15] and HSVd[6,17], known to infect pear. In the investigation, we have detected isolates of pear blister canker viroid (PBCVd)[1,3,5] infecting European pears cultivated in Japan. In this paper, we will report the detection, nucleotide sequence and diagnosis of the viroid, as well as discuss the possible relationship between the viroid and dimple pit and rough bark diseases of European pears (cv. La France) cultivated in

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Yamagata and Fukushima prefectures in Japan.

MATERIALS AND METHODS

Pear sources and extraction of low molecular weight RNA Fruit, leaves and bark of European pears (*Pyrus communis* L. var. *sativa* de Candolle) with or without rough bark symptoms on twigs (Sohi-byo) were collected from Fukushima prefecture[9-12]. Those of European pears with or without showing symptoms of dimple pit (Kubomika-byo)[13,14] and/or rough bark were collected from Yamagata prefecture. Those of Japanese pears (*P. serotina* Rehder var. *culta* Rehder) which showed positive or negative reactions to rough bark examination by grafting[12] were collected from Fukushima prefecture. Low molecular weight RNA (LMW-RNA) was then extracted from leaves or barks of pear as described by Li *et al.*[7]

Bioassay Because Li *et al.*[6] reported the presence of an isolate of HSVd from European pear cultivated in Japan, we have inoculated cucumber seedlings with 30 samples of LMW-RNA extract from pears and maintained in a greenhouse, 25-32°C with a 16 hr day length and light supplement for at least 6 weeks.

Polyacrylamide gel electrophoresis The LMW-RNAs extracted from 1-2 g of pear leaf tissue were run on return polyacrylamide gel electrophoresis (return-PAGE) followed by silver staining as described by Singh & Boucher[18], then on 5% PAGE containing 8M urea under denaturing conditions as described by Sano *et al.*[16]

Molecular cloning

(a) Cloning by Gubler & Hoffman Methods[4] Double stranded (ds) cDNA to a viroid-like RNA isolated from pear was synthesized using a cDNA synthesis kit (Takara Biochemicals), using the return-PAGE purified circular RNA as template. The ds-cDNA of blunt ended was ligated (DNA ligation kit, Takara Biochemicals) into Sma I site of pBluescript II SK(−) (Stratagene) and transformed *E.coli* (JM109).

(b) Cloning by reverse transcriptase (RT)—polymerase chain reaction (PCR) method On the basis of the partial nucleotide sequence obtained from a cDNA clone synthesized above, two sets of PCR primers were synthesized, that is, set 1 (5′-AGACCCTTCGTCGACGACGA-3′ and 5′-TGTCCCGCTAGTCGAGCGGA-3′) and set 2 (5′-CGCCGCGGTAAACTTCCA-3′ and 5′-ACTTGTGGTTCCTGTGGT-3′). The first strand cDNA was synthesized as above according to the manufacturer's instruction. Two μl of the resulting RT mixture was put in a PCR mixture (final volume of 50 μl) containing primers (20 pmole each) of set 1 or 2, 0.2 mM dNTPs and 1.25 U Tth DNA polymerase (Funakoshi). PCR was performed by 25 cycles of 1 min-denaturation at 94°C, 2 min-annealing at 55°C, and 3 min-primer extension at 72°C. PCR products were ligated into pGEM-T vector (Promega Corporation) and used for transformation of *E.coli* (JM109).

Hybridization for detection of viroids The same nucleic acid preparations used for the return-PAGE were re-examined by molecular hybridization. Hybridization was performed using DIG-labeled cRNA probes for apple scar skin viroid (ASSVd) and for the viroid-like RNA from pear as described by Li *et al.*[7]. The ASSVd cRNA probe was prepared as in Li *et al.*[7]. The cRNA probe for the viroid-like RNA from pear, which we identified as PBCVd later in this experiment, was prepared from a recombinant pGEM-T vector (pGEM-PBCV #16-13) containing a cDNA (about 300 nucleotides) of the viroid-like RNA found in sample no. 1 in Table 1.

Nucleotide sequencing Sequencing was performed using the standard dideoxynucleotide chain termination method, employed in a DIG Taq DNA sequencing kit (Boehringer Mannheim Biochemica) for manual sequencing and an AutoCycle sequencing kit for the ALFred DNA sequencer (Pharmacia Biotech).

RESULTS

Detection of a viroid-like RNA by PAGE analysis

A viroid-like RNA was detected from three of 37 samples examined by return-PAGE gel (Table 1, no. 1-3 ; Fig. 1a, lanes 1 and 3). The viroid-like RNA found in sample no.1 (Table 1, no. 1 ; Fig. 1a, lane 1) was recovered from the return-PAGE and again electrophoresed in a 5% PAGE containing 8 M urea, with known viroids as standards. The circular molecules of the viroid-like RNA in sample no.1 migrated faster than that of ASSVd (330 nucleotides), but slower than that of hop stunt viroid (HSVd ; 297 nucleotides) (Fig. 1b).

Cloning and partial nucleotide sequencing of viroid-like RNA in sample no.1 (Fukushima isolate)

Several cDNA clones were obtained from the viroid-like RNA in sample no.1 by Gubler & Hoffman method[4]. Partial sequencing data for one of the cDNA clones revealed that the sequence was highly homologous to no. 258-no. 50 of pear blister canker viroid (PBCVd) reported in France[5]. We have synthesized PCR primers (set 2 in Materials & Methods) based on the partial sequence data and used them for RT-PCR. A DNA fragment of *ca.* 280 bp was amplified using the primers, suggesting that the viroid-like RNA in sample no. 1 was closely related to PBCVd. The DNA fragments amplified were cloned into pGEM-T vector. One of the resulting cDNA clones, named pGEM-PBCV #16-13, was selected for preparing a DIG-labeled cRNA probe for the following hybridization assay.

Detection of PBCVd-like viroid, ASSVd and HSVd in pears

a) PBCVd-like viroid and ASSVd-hybridization assay using DIG-labelled cRNA probes Since the cRNA probe for PBCVd-like viroid was prepared from sample no.1 in Fukushima, the probe hybridized well

Table 1. Detection of PBCVd-like viroid, ASSVd and HSVd from pears collected in Fukushima and Yamagata prefectures using return-PAGE, cucumber assay and molecular hybridization

No.	Prefecture / Pear type / Cultivar	Disease symptoms	Return-PAGE	Cucumber assay HSVd	Hybridization ASSVd	Hybridization PBCVd	
	Fukushima						
	European pear						
1	Max Red Bartlett	(rough bark)[a]	+	−	−	+	sequenced
2	Max Red Bartlett	(rough bark)[a]	+	−	−	+	
3	seedling (FLF6020)	rough bark[b]	+	−	−	+	
4	seedling (FLF6020)	rough bark[b]	−	−	−	+	
5	La France	rough bark[b]	−	−	−	+[c]	
6	La France	rough bark	−	NT	−	−	6/12(50%)[e]
7	La France	rough bark	−	NT	−	−	
8	La France	rough bark	−	NT	−	+[c]	
9	Max Red Bartlett	(rough bark)[a]	−	NT	−	−	
10	Marguerite Marillat	(rough bark)[a]	−	NT	−	−	
11	Marguerite Marillat	(rough bark)[a]	−	NT	−	−	
12	Comice	rough bark	−	NT	−	−	
13	seedling (FLF6020)	healthy	−	−	−	−	
14	seedling (FLF6011)	healthy	−	−	−	−	0/3(0%)[e]
15	seedling (M12)	healthy	−	−	−	−	
	Japanese pear						
16	Sasaya	(rough bark)[a]	−	−	−	−	
17	Nijisseiki	(rough bark)[a]	−	−	−	−	
18	Nijisseiki	(rough bark)[a]	−	−	−	−	0/5(0%)[e]
19	Housui	(rough bark)[a]	−	−	−	−	
20	Housui	(rough bark)[a]	−	−	−	−	
21	Housui	healthy[a]	−	−	−	−	
22	Nijisseiki	healthy[a]	−	−	−	−	0/3(0%)[e]
23	Kousui	healthy[a]	−	−	−	−	
	Yamagata						
	European pear						
24	La France	dimple pit	−	−	−	+	
25	La France	dimple pit	−	−	−	−	
26	La France	dimple pit[d]	−	−	−	−	
27	Le Lectier	dimple pit[d]	−	−	−	−	3/8(37.5%)[e]
28	Marguerite Marillat	dimple pit[d]	−	−	−	−	
29	Beurre Bosc	(dimple pit)[d]	−	−	−	+	
30	Bartlett	(dimple pit)[d]	−	−	−	+	sequenced
31	Winter Nelis	(dimple pit)[d]	−	−	−	−	
32	Passe Crassane	healthy	−	−	−	−	
33	Silver Bell	healthy	−	−	−	−	
34	La France	healthy	−	−	−	−	0/6(0%)[e]
35	Le Lectier	healthy	−	−	−	−	
36	Bartlett	healthy	−	−	−	−	
37	Winter Nelis	healthy	−	−	−	−	

a) Parentheses indicate symptomless infection of rough bark agent. The infection of rough bark agent was examined by grafting a woody indicator clone (FLF6020)[12], which showed typical rough bark symptoms when grafted onto a tree carrying rough bark agent.

b) Sample nos. 3 and 4 were grafted directly onto sample nos. 1 and 2, respectively, and showed typical rough bark symptoms. Sample no. 5 was collected from a pear tree which was topworked by the scions taken from sample no. 1.

c) Hybridization was positive for PBCVd-like viroid, but the signal was very faint.

d) Sample nos. 26, 27, 28, 29, 30 and 31 were top-worked onto a pear tree (cv. Max Red Bartlett) which carries graft-transmissible dimple pit agent. Parentheses indicate symptomless infection.

e) Number of plants positive for PBCVd-like viroid/total examined by hybridization (percent positive)

with sample no.1 (Fig. 2). In addition to sample no.1, eight of the 36 samples showed positive reactions, indicating that a PBCVd-like viroid had also infected European pear samples nos. 2, 3, 4, 5, 8 from Fukushima and nos. 24, 29, 30 from Yamagata (Table 1). All the samples positive for PBCVd-like viroid were also posi-

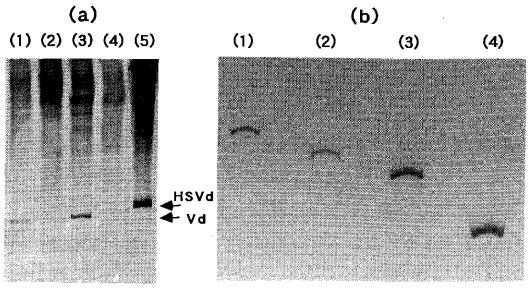

Fig. 1. (a) Return-PAGE of 2M LiCl-soluble RNAs extracted from pears with or without rough bark disease in Fukushima prefecture. Lanes (1) Max Red Bartlett (no. 1 in Table 1), (2) seedling (no. 13 in Table 1), (3) Max Red Bartlett (no. 2 in Table 1), (4) seedling (no. 14 in Table 1) and (5) HSVd-hop in cucumber. Arrows indicate a viroid-like RNA from pear (Vd) and HSVd.
(b) Co-electrophoresis of a circular molecule of a viroid-like RNA isolated from European pear [lane (2)] in Fukushima, with corresponding molecules of ASSVd [lane (1), 330 nucleotides], HSVd-hop [lane (3), 297 nucleotides] and HLVd [lane (4), 256 nucleotides] in 5% PAGE containing 8 M urea denaturing condition. The sample in lane (2) was a viroid-like RNA (no. 1 of Table 1) recovered from return-PAGE [Fig. 1(a), Vd].

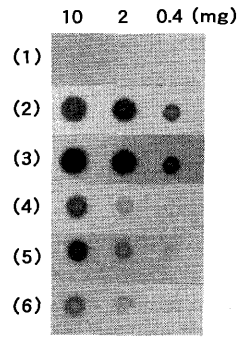

Fig. 2. Detection of PBCVd by hybridization using a DIG-labeled PBCVd cRNA probe. The figure shows only representative data from Table 1, that is, (1) no. 13, (2) no. 1, (3) no. 2, (4) no. 29, (5) no. 30 and (6) no. 24. Nucleic acids extracted from 10 mg of pear leaf tissues was serially diluted by five-fold up to 0.4 mg. Either 10, 2 or 0.4 milligrams of original tissues were applied in a 2 μl sample.

tive for dimple pit and/or rough bark agents as indicated by disease symptoms or graft-indexing (Table 1). The positive signals obtained from sample nos. 24 (cv. La France), 29 (cv. Beurre Bosc) and 30 (cv. Bartlett) suggested that the concentrations of PBCVd-like viroid in European pears were variable depending on the cultivars, and that the concentrations of the viroid in these samples were five to 25 times lower than those in sample nos. 1 and 2 (both cv. Max Red Bartlett) (Fig. 2), which may account for its lack of detection in these samples by return-PAGE analysis.

On the other hand, none of the Japanese pears tested positively to the PBCVd-like viroid probe, although some of them were positive to rough bark agent by graft-indexing. All the samples were negative to ASSVd (Table 1).

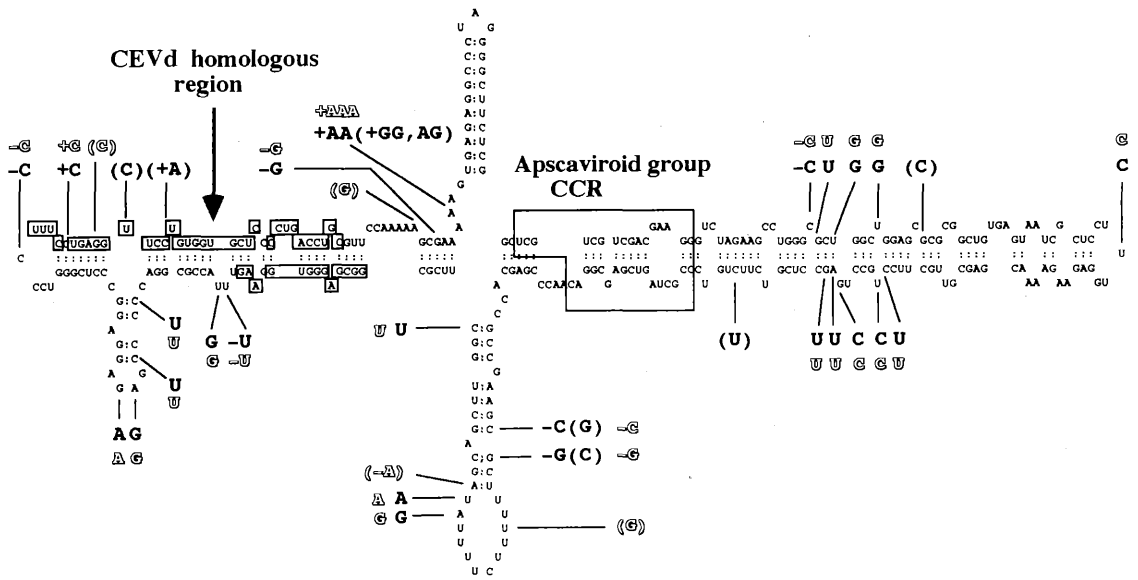

Fig. 3. Comparison of nucleotide sequences from a viroid-like RNA isolated from European pears in Japan (Fukushima, no. 1 and Yamagata, no. 30) and PBCVd in France to the predicted secondary structure model of PBCVd in France[5]. The sequences changed in Fukushima (black letters) and Yamagata (white letters) isolates were plotted around the PBCVd secondary structure. Minus marks (−) mean deletions and plus marks (+) mean insertions. The sequences in parentheses indicate minor sequence variations. Two regions in consensus with the central conserved region of ASSVd group (Apscaviroid group) and the right hand portions of CEVd are boxed.

b) HSVd-cucumber assay No HSVd infection was observed, suggesting that the pear samples examined in this experiment were not infected with HSVd. The results also indicate that the PBCVd-like viroid does not infect cucumber (Table 1).

Complete nucleotide sequencing of PBCVd-Fukushima (sample no. 1) and -Yamagata (sample no. 30) isolates

Because of the partial sequencing data of the viroid-like RNA in sample no. 1 and speculation that the viroid detected by hybridization from various pear samples is closely related to PBCVd, we synthesized two sets of primer (set 1 & 2 in Materials & Methods) for RT-PCR. To compare nucleotide sequences of PBCVd-like viroid isolated from European pears in Fukushima and Yamagata, RT-PCR was performed using partially purified preparations from sample no. 1 (cv. Max Red Bartlett) for Fukushima and no. 30 (cv. Bartlett) for Yamagata. Since sample no. 30 was taken from a Bartlett cultivar which was experimentally top-worked on a Max Red Bartlett carrying dimple pit agent, both of the isolates to be sequenced originated from the same cultivar Max Red Bartlett.

DNA fragments of *ca.* 230 bp and *ca.* 280 bp were amplified from both of the isolates using set 1 and set 2 primers, respectively. They were ligated to pGEM-T vector which produced numerous cDNA clones.

The complete nucleotide sequences of the viroid were determined by sequencing five individual cDNA clones for the Fukushima isolate and six clones for the Yamagata isolate. The sequences consisted of 312 nucleotides in the Fukushima isolate and 313 in the Yamagata isolate. The only difference between the consensus sequences of the two isolates was an A addition in the Yamagata isolate at the site between nos. 54 and 55 in the Fukushima isolate (Fig. 3). Sequence variants were observed at 12 positions in six of the 11 clones sequenced (Fig. 3).

DISCUSSION

The viroid detected in European pears cultivated in Japan consisted of 312 nucleotides in the Fukushima isolate and 313 in the Yamagata isolate, and showed 91.4-91.7% sequence homology with PBCVd from France (P2098T isolates)[5], indicating that they are isolates of PBCVd. The Fukushima isolate differed from the French isolate of PBCVd (P2098T) at 26 sites in the sequence, the Yamagata isolate at 27 sites. These changes include 17 base exchanges and six deletions for both isolates and three insertions for the Fukushima isolate and four for the Yamagata isolate. These differences, shown in Fig. 3 on the predicted secondary structure model by Hernandez *et al.*[5], are clustered mainly on the left terminal, the middle of the right terminal (the "variable" region) and the upper and lower branches on the left side (the "pathogenicity" region) of the central conserved region (CCR). No sequence heterogeneity was found in the CCR typical for Apscaviroid group (Fig. 3, boxed sequence). The overall sequence conservation/variation pattern found in PBCVd is consistent with other viroids analyzed so far. That is, PBCVd has both a "variable region" and a "pathogenicity region", an A-rich sequence in the upper strand and a U-rich sequence in the lower strand, although the corresponding region branched out in this secondary structure model. One exception in this viroid was the relatively high rate of variations found in the left terminal region, as described above.

Recently, Ambros *et al.*[1] reported sequence variations in French isolates of PBCVd (isolates P1914T and P47A). We also found three (nos. 50, 149 and 176) of the six variations observed in isolates P1914T and P47A, but the other three (nos. 119, 234 and 236) were not found in the Japanese isolates. Furthermore, only one (no. 24) of the 12 minor sequence variations in the French isolates was consistent with the variations found in Japanese isolates. These results clearly indicate that the Japanese isolates (Fukushima and Yamagata) are related more closely with each other than with any other isolates in Europe. The two Japanese isolates may have originated from the same contaminated sources, although they are now cultivated separately in different prefectures in Japan. Interestingly, as described in the results, they both were isolated from the cultivar Max Red Bartlett.

Possible relationship between PBCVd and rough bark (Sohi-byo) and dimple pit (Kubomika-byo) on European pear cultivated in Japan

Our surveys by molecular hybridization using PBCVd and ASSVd probes, and by HSVd-cucumber bioassay, indicated that PBCVd was detected from 50% (6/12) of European pears carrying the rough bark agent (indexed by grafting) in Fukushima, and from 37.5% (3/8) of European pears carrying dimple pit and/or rough bark agents in Yamagata. On the other hand, the agent was not detectable from either of five Japanese pear samples carrying rough bark agent or all nine healthy European and Japanese pear samples examined.

The relationship between the diseases and PBCVd is still unclear ; our PBCVd indexing by hybridization was not completely consistent with the results obtained by symptom- or graft-indexing of rough bark/dimple pit diseases (Table 1). However, we obtained a higher detection rate (9/20=45%) of PBCVd in European pears affected by the rough bark/dimple pit agent as compared to Japanese pears (0/8=0%) and healthy European pears (0/9=0%). Moreover, the descriptions of blister canker disease[19] of cultivated pears in Europe, of which the French isolate of PBCVd is a causal agent, and rough bark disease[11,13] in Japan are quite similar, although no comparative research has been done. Furthermore, Ambros *et al.*[2] reported that similar bark disorders occurring on the pear cultivar Williams (Bartlett in Japan) were negative to PBCVd, suggesting that a rough bark agent of different etiology exists. This is

consistent with our result that not all of the rough bark samples tested positive to PBCVd. By considering these results, we may conclude that PBCVd is associated with rough bark/dimple pit diseases occurring in European pears cultivated in Japan, at least of those on La France originated from Max Red Bartlett[11,13,14]. Further investigations are definitely required to examine the infection of PBCVd in various sources of pear trees. Back-inoculation experiments with purified PBCVd preparations onto pear seedlings are now in progress.

Li *et al.*[6,17] reported an isolate of HSVd from European pear cultivated in Japan. Osaki *et al.*[15] isolated ASSVd from Japanese pear and reported that ASSVd was a causal agent of fruit disorder occurring on Japanese pears (cv. Niitaka). Our results, however, clearly indicate that neither HSVd nor ASSVd is a causal agent of rough bark and/or dimple pit diseases of European pears cultivated in Japan.

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和 文 摘 要

佐野輝男・李 世訪・尾形 正・落合政文・鈴木千代吉・大沼幸男・四方英四郎：日本産セイヨウナシから分離されたナシblister canker ウイロイドについて

日本(福島県と山形県)で栽培されているセイヨウナシからウイロイドを検出した．塩基配列を解析した結果，フランスでセイヨウナシからの分離が報告されている pear blister canker viroid(PBCVd)と同定された．福島分離株は312塩基，山形分離株は313塩基よりなり，両分離株間の違いは僅か1カ所で，福島分離株の54番目と55番目の塩基の間に山形分離株では一塩基余分にAが付加されていた．フランス分離株(P2098T, Hernandez *et al.*, 1992)と比較して福島分離株では塩基置換17カ所，挿入3カ所，欠失6カ所の合計26カ所，山形分離株ではさらに挿入1カ所の合計27カ所で変異が認められた．本ウイロイドと福島県および山形県で発生が報告されているセイヨウナシ粗皮病，くぼみ果病との関係を検討した結果，福島県から採集した粗皮病罹病セイヨウナシでは50%(6/12)また山形県から採集したくぼみ果病罹病セイヨウナシでは37.5%(3/8)からPBCVdが検出され，共に健全セイヨウナシの0%(0/9)と日本ナシの0%(0/8)に比べ明らかに高い検出率を示した．