Seed- and Pollen-transmitted Double-stranded RNAs Detected from Japanese Pear*

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Abstract

Three nucleic acids were found in 5% polyacrylamide gel electrophoresed with extracts from several cultivars of Japanese pear (*Pyrus* spp.) that had no symptoms. They were cultivar-specific, and were detected from bark, leaf, petals and flowers plus petals. They were not digested by DNase I and were resistant to RNase A in $2\times$ SSC buffer, but were susceptible to RNase A in $0.1\times$ SSC buffer. These nucleic acids were absorbed by CF-11 cellulose in 15% ethanol. These results indicate that the three nucleic acids were double-stranded (ds) RNA. Molecular weights were estimated at about $1.08, 0.97, 0.90\times 10^6$ daltons according to electrophoresis with dsRNAs of the rice dwarf virus as a marker. Several cultivars that have these dsRNAs had genetic relationships. The results of investigating the transmission of dsRNAs to the next generation by crossing showed that dsRNAs were detected in 31/40 when only the seed parent had dsRNAs, in 12/12 when only the pollen parent had dsRNAs, and in 5/5 when both parents had dsRNAs. These dsRNAs were transmitted high frequently to the next generation through both ovule and pollen, but transmission of dsRNAs by grafting to other cultivars without dsRNAs did not occur. These results suggest that the dsRNAs from Japanese pear are similar to dsRNAs derived from the phyto-cryptovirus group.

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Key words : dsRNA, seed-transmission, pollen-transmission, cryptic virus, Japanese pear.

INTRODUCTION

During a survey for various virus-like diseases of Japanese pear, we found three double-stranded (ds) RNAs in extracted nucleic acids. But healthy trees also had these dsRNAs. They were not related to diseases, but were cultivar-specific.

The dsRNAs are not normally found in plants unless they are infected with RNA virus. However, dsRNAs have been discovered in various plants without symptoms, from algae⁽¹⁰⁾ to higher plants. These dsRNAs have two groups: 1) low-molecular weight dsRNAs (from 1.2 to 2.5 kb) that are related to virus-like particles, called cryptic viruses; and 2) high-molecular weight dsRNAs (more than 10 kb) that are not associated with virus-like particles. The latter also includes two groups: ① dsRNAs found from *Vicia faba*^(8,14), sunflower⁽²⁾, maize⁽²³⁾ and rice⁽²⁷⁾ plants that are thought to be associated with cytoplasmic male sterility (CMS), and ② endogenous dsRNAs found from *Phaseolus vulgaris*^(18,26), barley⁽²⁹⁾, pepper^(24,25), alfalfa⁽⁵⁾, rice⁽⁶⁾ and cassava⁽⁷⁾, whose origins and biological functions are unknown. The properties of the latter are: (1) no symptoms in their host plants, (2) seed- and pollen-mediated

inheritance (no transmission via grafting), (3) no distinct virus-like particles, and (4) a constant concentration in their host cells.

Cryptic viruses are known from alfalfa⁽²²⁾, beet^(11,12,21,22,28), carnation^(15,16), hop trefoil⁽¹⁷⁾, radish^(19,20), poinsettia⁽¹³⁾, ryegrass⁽³⁾, spinach⁽²¹⁾, vicia⁽¹⁾, white clover⁽²²⁾ and so on. The properties of cryptic viruses are: (1) isometric particles, (2) the nucleic acid consists of segmented dsRNA, (3) no symptoms in their host plants, (4) low concentration in infected plants, (5) high frequency of seed- and pollen-mediated transmission⁽¹³⁾ (no transmission via grafting), (6) a constant concentration in their host cells.

In this study, we investigated the detection of dsRNAs, which are similar to dsRNAs of cryptoviruses, in several cultivars of Japanese pear (*Pyrus* spp.). We also describe some properties of the dsRNAs.

MATERIALS AND METHODS

Source of material All cultivars of Japanese pear used in this research are maintained in the National Institute of Fruit Tree Science. But three cultivars, cvs. Niitaka, Nansui and Okusankichi, were sampled at several places (Table 2).

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Purification of dsRNAs Each gram of tissue was homogenized by a polytron with 1.8 ml of 2×STE buffer (0.1 M NaCl, 50 mM Tris, 1 mM Na₂EDTA, pH 7.0), 0.2 ml of 10% SDS, 2 μ l of mercaptoethanol, 1 ml of chloroform and 1 ml of water-saturated phenol containing 0.1% 8-hydroxyquinoline. After the homogenate was centrifuged, the upper aqueous phase was collected, adjusted to 15% ethanol. To each 10 ml of the 15% ethanol solution, 0.5 g CF-11 (Whatman) cellulose powder⁴⁾ was added. The mixture was contained in small columns and was washed with a solution of 15% ethanol-STE buffer. The dsRNAs were then eluted from the CF-11 cellulose with 100% STE buffer and were precipitated by adding 2.5 parts of cold ethanol. The precipitate was collected by centrifugation, and was dissolved in a loading buffer (0.05% bromophenol blue, 5% glycerol) for electrophoretic analysis.

Electrophoresis Purified dsRNAs were analyzed by electrophoresis in a 5% polyacrylamide gel in TAE buffer (0.04 M Tris, 0.02 M acetic acid, 1 mM Na₂EDTA, pH 7.4) at 75 V for 17 hr. Gels were stained with ethid-

ium bromide, and nucleic acids were visualized on an UV transilluminator and were photographed using Polaroid 667 film. The dsRNAs of rice dwarf virus (RDV), provided kindly by Dr. Omura of the National Agriculture Research Center, and radish yellow edge virus (RYEV), extracted from radish cv. Minowase, were used as molecular markers.

Digestion by DNase I and RNase A In the DNase I treatment, the extracted nucleic acids were suspended in 150 μ l of TAE buffer containing 5 mM MgCl₂. The suspensions was added 5 μ l of DNase I (10 units/ μ l, RNase-free, Boehringer Mannheim Biochemica), and were incubated at 37°C for 1 hr. For the RNase A treatment, the extracted nucleic acids were suspended in 400 μ l of 2× or 0.1×SSC (1×SSC; 0.15 M NaCl, 0.015 M sodium citrate). The suspensions were added 4 μ l of RNase A (1 mg/ml, Boehringer Mannheim Biochemica), and were incubated at 37°C for 30 min. After incubation, the reaction mixtures were precipitated with ethanol, and centrifuged. The pellets were dissolved in a loading buffer for electrophoresis.

Table 1. Detection of dsRNAs in cultivars of pear

Cultivar	Detection of dsRNAs	Cultivar	Detection of dsRNAs
<i>Pyrus pyrifolia</i> Nakai var. <i>culta</i> Nakai		<i>Pyrus hondoensis</i> Nakai et Kikuchi	
Abumi	+ ^{a)}	Aonashi-Fujisanroku	—
Amanogawa	+	<i>Pyrus betulaefolia</i> Bunge	
Atago	—	Hokushi Mamenashi	—
Chikusui	—	Kara Mamenashi	—
Choujuuro	—	Manshuu Mamenashi	—
Doitsu	—	<i>Pyrus calleryana</i> Decne. (var. <i>dimorphophylla</i> Koidz.)	
Echigonishiki	+	Aichi Mamenashi	+
Hakuteiryuu	—	Inunashi	—
Housui	—	Mamenashi 4	+
Imamuraaki	—	Mamenashi 5	—
Inagi	—	Mie Mamenashi 39	—
Ishiiwase	—	Mie Mamenashi 46	—
Kikusui	—	<i>Pyrus uyematsuana</i> Makino	
Kimizukawase	—	Ainashi	—
Kinchaku	—	<i>Pyrus calleryana</i> Decne. var. <i>fauriei</i> Nakai	
Kousui	—	Chousen Mamenashi	—
Kumoi	—	<i>Pyrus pashia</i> Buch. Ham.	
Kunitomi	+	Nepal Pear A	—
Niigatanashi	—	Hybrid (Okusankichi × La France)	
Nijisseiki	—	Ooharabeni	—
Niitaka	+	Taiheiyuu	—
Nansui	+	Others (History unknown varieties, clones)	
Okusankichi	+	Akita Tazawa 3 Gou	+
Shinchuu	—	Saitama 2-1	—
Shinkou	—	Taiwan Nashi D	—
Shinsei	—	<i>Pyrus communis</i> L.	
Shinsetsu	+	La France	—
Shinsui	—	Max Red Bartlett	—
Taihaku	—	Winter Nelis	—
Yanaga	+		
Yokogoshi	—		
Yoshino	—		

a) + : detected, — : not detected.

Transmission of dsRNAs by grafting Scions of one cultivar that had dsRNAs were grafted onto another cultivar that had no dsRNAs, and, conversely, scions of a cultivar that had no dsRNAs were grafted onto another cultivar that had dsRNAs. Bark samples were collected four years after grafting. The dsRNAs were purified and electrophoresed.

Crossing At the onset of flowering, the inflorescences of paired plants were enclosed in a sealed, pollen-proof paper bag to exclude extraneous pollen, and to allow interpollination. Table 4 shows the combinations of paired plants. Seeds were removed from ripe fruits, were incubated at 4°C for a few months to break the dormancy of the seed, and then were sowed. Three lines, 162-29 (with dsRNAs), HG-7 (with dsRNAs) and HG-8 (without dsRNAs), and two cultivars, cvs. Shinsei (without dsRNAs) and Okusankichi (with dsRNAs), were used.

RESULTS

Detection of dsRNAs from Japanese pear trees

Three nucleic acids were found in 12 of the 53 cultivars of pear without any symptoms (Table 1) and from all trees of three cultivars (cvs. Niitaka, Nansui, Okusankichi) sampled at several places (Table 2). They were detected from bark, leaf, flowers plus petals and petals only (Fig. 1).

Digestion by DNase I and RNase A

The nucleic acids were not digested by DNase I and were resistant to RNase A in 2×SSC buffer, but were susceptible to digestion by RNase A in 0.1×SSC buffer (Fig. 2). These nucleic acids were absorbed by CF-11 cellulose in 15% ethanol (Fig. 3). These results indicate that the three nucleic acids are double-stranded RNA.

Estimation of dsRNA molecular weights

All three dsRNAs found in several cultivars had the same mobilities in electrophoresis (Fig. 4). The molecu-

Fig. 1. Electrophoresis of dsRNAs isolated from different parts of the Japanese pear plant (cv. Niitaka) in 5% polyacrylamide gel. Lane M, RDV dsRNA as marker. Preparations from bark (lane 1), petal (lane 2), flower including petals (lane 3), leaf (lane 4). Arrows indicate the positions of dsRNAs.

Fig. 2. Polyacrylamide gel electrophoresis of dsRNAs isolated from Japanese pear after treatment with DNase I and RNase A. Lane 1, *Hind*III-digested λ ; lane 2, untreated dsRNAs; lane 3, *Hind*III-digested λ treated with DNase I; lane 4, dsRNAs treated with DNase I. Lane C, untreated dsRNAs; lane H, dsRNAs treated with RNase A in 2×SSC; lane L, dsRNAs treated with RNase A in 0.1×SSC. Arrows indicate the positions of dsRNAs.

Table 2. Detection of dsRNAs in Japanese pear cultivars Niitaka, Nansui and Okusankichi sampled at several areas in Japan

Cultivar	Sampling place	No. of detected plants/ No. of examined plants
Niitaka	Chiba	8/8
	Oita	4/4
	Ibaraki	2/2
	NIFTS ^{a)}	2/2
Nansui	Nagano	26/26
	NNAES ^{b)}	1/1
	NIFTS	1/1
Okusankichi	Okayama	2/2
	Kumamoto	2/2
	Oita	1/1
	NIFTS	1/1

a) NIFTS: National Institute of Fruit Tree Science.

b) NNAES: Original seedling of Nansui at Nagano Nanshin Agricultural Experiment Station.

Fig. 3. Analysis of the binding ability of dsRNAs isolated from Japanese pear to CF-11 cellulose by polyacrylamide gel electrophoresis. After SDS-phenol extraction, the aqueous phase (lane 1), washed elute (15% ethanol solution) from CF-11 (lane 2), and final elute (STE buffer) from CF-11 (lane 3), respectively, were precipitated with ethanol. The precipitates that formed were collected by centrifugation, and dissolved in a suitable buffer for electrophoretic analysis. Arrows indicate the positions of dsRNAs.

Fig. 4. Polyacrylamide gel electrophoresis of dsRNAs isolated from Japanese pear. Lane M, RDV dsRNA as marker; lane 1, Aichi Mamenashi; lane 2, radish cv. Minowase; lane 3, Niitaka; lane 4, Nansui; lane 5, Okusankichi; lane 6, Yanaga; lane 7, Echigonishiki; lane 8, Mamenashi 4; lane 9, Akita Tazawa 3 Gou. Arrows indicate the positions of dsRNAs. Three bands in lane 2 were dsRNAs from radish yellow edge virus, a cryptovirus.

Fig. 5. Genetic relationship of Japanese pear cultivars and transmission of dsRNAs by crossing. ♂ and ♀, pollen parent and seed plant. + and -, with and without dsRNAs. NT, not tested.

lar weights were estimated at about $1.08, 0.97, 0.90 \times 10^6$ daltons according to electrophoresis with the dsRNAs of RDV and RYEV as markers.

Detection of dsRNAs from various cultivars of *Pyrus* spp.

Several cultivars of Japanese pear had dsRNAs (Table 1), that had a genetic relationship with each other (Fig. 5). The dsRNAs were found in three wild varieties, Aichi Mamenashi, Mamenashi 4 and Akita Tazawa 3 Gou. European pears and the other pears did not have dsRNAs. Two other dsRNAs were discovered in Akita Tazawa 3 Gou, indicated by arrows above the dsRNAs (Fig. 4). Their molecular weights were estimated at about $1.35, 1.31 \times 10^6$ daltons, when the dsRNAs of the RDV were used as molecular weight markers, but they were not investigated in detail in this study.

Transmission of dsRNAs by grafting

Transmission of dsRNAs by grafting to other cultivars without dsRNAs did not occur (Table 3).

Transmission of dsRNAs to the next generation by crossing

Transmission of dsRNAs to the next generation by crossing was investigated. The dsRNAs were detected in 31 of 40 seedlings when only the seed parent had dsRNAs, in 12 of 12 seedlings when only the pollen parent had dsRNAs, and in 5 of 5 seedlings when both parents had dsRNAs. Thus dsRNAs were transmitted high frequently to the next generation through both ovule and pollen (Table 4).

Table 3. Transmission test of dsRNAs by grafting

Scion/Stock	Detection Scion/Stock	Transmission
Chikusui (–) ^{a)} / Okusankichi (+)	–/+ ^{b)}	–
Okusankichi (+)/ Chikusui (–)	+/–	–
Nansui (+)/ Chikusui (–)	+/–	–
Nijisseiki (–)/ Niitaka (+)	–/+	–
Niitaka (+)/ Nijisseiki (–)	+/–	–

a) (+) : inoculum with dsRNAs, (–) : inoculated plants without dsRNAs.

b) + : dsRNAs were detected, – : dsRNAs were not detected.

DISCUSSION

This study showed that dsRNAs extracted from Japanese pear have the properties of (1) high frequency of seed- and pollen-transmission, (2) untransmissibility by grafting, (3) causing no symptoms and (4) low-molecular weight dsRNAs. These properties are similar to those of phyto-cryptoviruses. We tested virus-like particles in dip and partially purified preparations of Japanese pear. But virus-like particles have not been found yet (unpublished results).

The subgroup of high-molecular weight dsRNAs is associated with cytoplasmic male sterility (CMS). But CMS is unknown in Japanese pear, and is known to be cross incompatible. These dsRNAs, however, do not seem to be related to cross incompatibility, because the presence of dsRNAs is not associated with cross incompatibility.

In this study, dsRNAs of Japanese pear were not transmitted by grafting, but were transmitted by seed and pollen. We suggest that these dsRNAs do not have the capability to move from cell to cell, and that they move to new cells by the cell divisions of the host. Hammer *et al.*⁹⁾ showed that beet cryptic viruses were useful as a breeding marker, and as a tool to characterize the germplasm, and to study the evolution of beets, because cryptoviruses induce no apparent symptoms and the only means of their transmission is by seed and pollen. The dsRNAs that we found in cultivated and wild pears also seem to be transmitted by seed and pollen. These results indicate that dsRNAs found in Japanese pears also could be used as a tool to characterize the germplasm and to study the evolution of Japanese pears. An interesting study would be the origins and biological functions of dsRNAs, the relationships between dsRNAs and cryptoviruses, and high-molecular weight dsRNAs.

Table 4. Transmission of dsRNAs to next generation by crossing

Seed parent × Pollen parent	No. of seedlings detected/ No. of seedlings tested
162-29 × Shinsei (+ × –) ^{a)}	31/40
HG-8 × Okusankichi (– × +)	12/12
HG-7 × Okusankichi (+ × +)	5/5

a) + : with dsRNAs, – : without dsRNAs.

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和 文 摘 要

大崎秀樹・佐藤義彦・工藤 晟：ニホンナシから検出された種子および花粉伝染性の2本鎖RNA

無病徴のニホンナシから抽出した核酸を5%ポリアクリルアミドゲル電気泳動により分析したところ、特定の品種から抽出した核酸にのみ特異的に共通して3本のバンドが認められた。これらの核酸は、樹皮、葉、花弁そして花弁を含む花から検出された。これらは、DNase Iによって消化されず、RNase Aに対しては、2×SSC下では消化されず、0.1×SSC下で消化された。さらに、15%エタノール存在下でCF-11 セルロースパウダーに吸着された。以上の結果から、3本のバンドは2本鎖(ds)RNAであることが判明した。イネ萎縮ウイルスのdsRNAをマーカーとした実験により、分子量はそれぞれ1.08, 0.97, 0.90×10⁶と計算された。また、dsRNAが検出された数品種は親子関係にあるため、交配による後代への伝染を調べた。その結果、dsRNAを種子親だけが持っている場合は40検体中31検体から、花粉親だけが持っている場合は12検体中12検体から、両方が持っている場合は5検体中5検体から、dsRNAが検出され、高い確率で後代に伝染することが判明した。しかし、本dsRNAsの接木伝染は認められなかった。これらの知見から、本dsRNAが種子伝染性潜伏ウイルス由来であることが示唆された。