Nucleotide Sequence of Seed- and Pollen-transmitted Double-stranded RNA, which Encodes a Putative RNA-dependent RNA Polymerase, Detected from Japanese Pear*

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The nucleotide sequence of the largest double-stranded (ds) RNA (named dsRNA1) of three species of seed- and pollen-transmitted dsRNA species detected from Japanese pear was analyzed, and one strand was found to contain a single long open reading frame (ORF) of 1434 nucleotides that encoded a putative polypeptide containing 477 amino acid residues with a molecular mass of 54.9 kDa. This polypeptide contained amino acid sequence motifs conserved in putative RNA-dependent RNA polymerases of RNA viruses. Attempts to visually identify or purify virus-like particles associated with the dsRNAs were unsuccessful. Slow sedimentation of the dsRNA fraction suggests that the dsRNAs may be unencapsidated. The concentration of dsRNAs in the host, Japanese pear, was about 16 times higher than that from a cryptic virus, radish yellow edge virus (RYEV). These results suggest that the dsRNAs were not from cryptic viruses. Partial nucleotide sequences of the two smaller dsRNAs (named dsRNAs 2 and 3) and two other dsRNAs (named dsRNAs 4 and 5) detected from only the Japanese pear cultivar (cv.) Akita Tazawa 3 Gou were analyzed, and encoded nearly the same amino acid sequence encoded by dsRNA1.

Key words: cryptic virus; dsRNA; RNA-dependent RNA polymerase

The presence of dsRNA in plant extracts is often an indicator of viral infection. With the exception of retroviruses, plus and minus sense single-stranded RNA viruses produce an RNA strand complementary to the genomic one during their replication. These two complementary strands form a double-stranded RNA structure. During a survey for various virus-like diseases of Japanese pear, we found three dsRNAs (dsRNAs 1, 2, and 3; about 1.08, 0.97, and 0.90 × 10⁶ daltons) in nucleic acids extracted from bark, leaf, flowers with petals, and petals only. But healthy trees also had these dsRNAs. They were not related to diseases, but were cultivar-specific. The properties of the dsRNAs were similar to dsRNAs of cryptoviruses. Two other dsRNA species (dsRNAs 4 and 5; 1.35 and 1.31 × 10⁶ daltons) were found in only cv. Akita Tazawa 3 Gou besides dsRNAs 1, 2, and 3, but dsRNAs 4 and 5 have not been investigated to find if they were seed- and pollen-transmitted.

Recently, dsRNAs have been discovered in various plants without symptoms, from algae to higher plants. These dsRNAs have two groups: low-molecular weight dsRNAs (from 1.2 to 2.5 kb) related to virus-like particles, called cryptic viruses; and high-molecular weight dsRNAs (more than 10 kb) not associated with virus-like particles. The latter also includes two groups: dsRNAs that are thought to be associated with cytoplasmic male sterility, and an endogenous dsRNA group, the origins and biological functions of which are unknown.

In this study, we analyzed the nucleotide sequence of one seed- and pollen-transmitted dsRNA species (dsRNA1) from Japanese pear and the partial nucleotide sequences of four other dsRNAs (dsRNAs 2, 3, 4, and 5), and we tried to visually identify or purify virus-like particles associated with the dsRNAs. Here, we also discuss possible functions of the encoded polypeptide.

Materials and Methods

Extraction of dsRNAs. Japanese pear cvs. Niitaka and Akita Tazawa 3 Gou are maintained in National Institute of Fruit Tree Science. The dsRNAs were purified from bark tissue of cv. Niitaka, dsRNAs 4 and 5 from bark tissue of cv. Akita Tazawa 3 Gou as described. The dsRNAs of RYEV were extracted from seedlings of radish cv. Minowase, the seeds of which were commercially obtained.

Electrophoresis and purification of dsRNAs. The dsRNAs were analyzed by electrophoresis in 5% polyacrylamide gel as described. For RT-PCR, cloning and dot blot, dsRNAs were purified twice by excising bands in gels after electrophoresis, and eluting. The dsRNAs were denatured, spotted, and hybridized as described.

Digoxigenin (DIG)-labeled 267-bp product amplified with primers 1h and 4c (for details, see below) from dsR-

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* The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB012616.

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Abbreviations: BCV3, beet cryptic virus 3; CTeV, carrot temperate virus; cv., cultivar; ds, double-stranded; ORF, open reading frame; RYEV, radish yellow edge virus
NA1 was used as a probe. DIG-labeling and detection were done by the protocol of a DIG DNA Labeling and Detection Kit (Boehringer Mannheim). RNA molecular weight marker III (Boehringer Mannheim) was spotted as a control.

**RT-PCR, cloning, and sequencing.** Three primers were designed according to three of the four nucleotide sequences of the beet cowpea virus (BCV) dsRNA2, which encode four conserved amino acid sequences of putative RNA-dependent RNA polymerases, and the nucleotide sequence of the carrot temperate virus (CTeV). The primers synthesized were 1h (5′-GCCGAATACACCATCCG-3′ corresponding to nucleotides from AGIPS in motif II of CTeV), 3h (5′-GATTGCTAAGTTTGAC-3′ corresponding to nucleotides from DWSSFD in motif I of BCV3), and 4c (5′-TGCCTTCTTCTAAGA-3′ complementary to nucleotides from FLGRTA in motif IV of BCV3). Also three primers, 6c (5′-ATAATAGTGTAAATAC-3′), 7h (5′-CCATTCGCAACGATAC-3′) and 8c (5′-GTAGACGTGTGACGAC-3′) were designed according to the sequences that had already been analyzed. The first strand cDNA was synthesized in 8.6 µl of RT reaction mixture containing 4 µl (one twentieth of the dsRNAs obtained from 5 g of bark tissue) of electrophoretically purified preparations denatured by boiling for 5 min at 100°C, 2 µM downstream primer, 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 10 mM dithiothreitol (DDT), 3 mM MgCl2, 0.5 mM each dNTP and 86 units of M-MLV reverse transcriptase (Gibco BRL). The reaction was incubated for 1 hr at 37°C. Ten µl of PCR mixture contained 2.5 µl of the RT reaction mixture, 1.2 µM upstream- and downstream-primers, 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM MgCl2, 0.1% Triton X-100, 0.2 mM each dNTP, and 0.5 units of Taq DNA polymerase (Toyobo Co., Ltd.). The mixture was layered with 50 µl mineral oil. All PCRs were done in a thermal cycler PC-700 (ASTEC), and were performed by 30 cycles of 30 sec of denaturation at 94°C, 30 sec of annealing at 62°C, and 45 sec of primer extension at 72°C, with a final extension at 72°C for 7 min. Samples (5 µl) of each RT-PCR product were analyzed by electrophoresis in 5% polyacrylamide gel in TBE buffer (89 mM Tris, 89 mM boric acid, 2.5 mM Na2EDTA, pH 8.3). Gels were stained with ethidium bromide.

Clones representing both ends of the dsRNAs were obtained by using the 5′ RACE System for Rapid Amplification of cDNA Ends, Version 2.0 (Gibco BRL) with primers 7h and 8c. All products were cloned using the TA Cloning Kit (Invitrogen). Two independent clones per product were sequenced. The DNA sequencing was analyzed in an automated DNA sequencer (model 377, Applied Biosystems). Both strands were sequenced at least once.

**Purification of viral particles.** Dip and partially purified preparations were observed by electron microscopy by the method of Natsuki.10 Bark tissue of cv. Niitaka was used for partial purification.

**Results and Discussion.**

**Nucleotide sequences of dsRNAs from Japanese pear.** We had thought that dsRNAs from Japanese pear were from cryptic viruses, because their properties were similar to those from cryptic viruses.11 Then we tried to amplify cDNA from the electrophoretically purified dsRNAs by RT-PCR with primers designed according to the conserved amino acid sequence motifs of RNA-dependent RNA polymerases in dsRNAs of BCV3 and CTeV. DNA products of expected size, 267 bp, were obtained from dsRNAs 1, 2, 3, 4, and 5, and a few nonspecific bands were visible in the lanes of dsRNAs 1 and 2, using the primer pair 1h and 4c (Fig. 1). The strategy for sequencing dsRNA1 was as follows. The products amplified with primers 1h and 4c, and with 3h and 6c were sequenced. Clones representing both ends of the dsRNAs were obtained by using the 5′ RACE System with primers 7h and 8c, respectively. The 3h primer did not anneal to the motif I region, but to the region shown in Fig. 2. The nucleotide sequence of cDNA of dsRNA1 from Japanese pear was analyzed and the corresponding plus strand RNA sequence is shown in Fig. 2. One long ORF of 1434 nucleotides was found that encoded a predicted polypeptide of 477 amino acids with a calculated molecular mass of 54.9 kDa. The conserved amino acid sequence motif of GDD (at position 354–356) was found in the deduced polypeptide (Fig. 2), suggesting that it is probably an RNA-dependent RNA polymerase. This was supported by the presence of the other three conserved amino acid sequence motifs also found.

![Fig. 1. Polycrylamide Gel Electrophoresis of DsRNAs from Japanese Pear and RT-PCR Amplified cDNA Products from Purified dsRNAs.](image-url)
Fig. 2. Nucleotide Sequence of dsRNA1 and Deduced Amino Acid Sequence.

The termination codon is indicated by an asterisk. The conserved amino acid sequence motifs are boxed. The arrowheads indicate the 3′ end of the region where the primers anneal.

<table>
<thead>
<tr>
<th>Motif I</th>
<th>Motif II</th>
<th>Motif III</th>
<th>Motif IV</th>
</tr>
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<tbody>
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<td><strong>BMV</strong></td>
<td><strong>Bacillibaculovirus</strong></td>
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<td>B</td>
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<td>BDIFPSDXQ</td>
<td>BIDFPSDXQ</td>
</tr>
<tr>
<td>C</td>
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Fig. 3. Conserved Amino Acid Sequences of Putative RNA Polymerase Encoded by RNAs of Plus-strand RNA Viruses, Cryptic Viruses and dsRNA Viruses.

Highly conserved residues are indicated by asterisks. A, plus-sense RNA viruses; B, dsRNA viruses. SNBV, Sindbis virus; AIMV, alfalfa mosaic virus; BMV, brome mosaic virus; BMSV, barley stripe mosaic virus; TRV, tobacco rattle virus; TMV, tobacco mosaic virus; BNYV, beet necrotic yellow vein virus; TYMV, turnip yellow mosaic virus; PVX, potato virus X; BCV3, beet cryptic virus 3; C. parvum, dsRNAs in Cryptoporidae parvum; Fusov, Fusarium solani virus; ROT, bovine rotavirus (L); RED, reovirus serotype 3 (L); BDV, bluetongue virus serotype 10 (L); RDV, rice dwarf virus (S); Scv, Saccharomyces cerevisiae virus; HAV, hypovirulence-associated virus; BDV, infectious bursal disease virus; B, bacteriophage φ6. Numbers indicate the number of conserved residues contained in each motif.
in genes encoding RNA-dependent RNA polymerases from other characterized single-stranded and dsRNA viruses (Fig. 3). A comparative analysis of the putative product of dsRNA1 with databases using BLAST programs found sequence similarity with RNA-dependent RNA polymerases from BCV dsRNA2, and dsRNAs in the parasitic protozoan Cryptosporidium parvum that infects the gastrointestinal tract of a variety of mammals, and a mycovirus of the plant pathogenic fungus, Fusarium solani (Fusov). In the four conserved motifs, the dsRNA1 from Japanese pear shared 67%, 58%, and 38% identity with BCV3, dsRNAs from C. parvum, and Fusov, respectively.

The 5′-terminal sequence AGAAUUU was found in dsRNA1. The 5′-terminal sequences of BCV3 dsRNAs 1 and 2 contained the same sequence, which suggests that dsRNA1 from Japanese pear is similar to BCV3 dsRNA2.

The DNA products were also amplified from dsRNAs 2, 3, 4, and 5 by two primers, 1h and 4c (Fig. 1). DIG-labeled 267 bp product amplified by 1h and 4c from dsRNA1 hybridized to gel-purified dsRNAs 2, 3, 4, and 5 (Fig. 4A). The dsRNAs 4 and 5 were found specifically in only Japanese pear cv. Akita Tazawa 3 Gou with three seed- and pollen-transmitted dsRNA species, dsRNAs 1, 2, and 3. But dsRNAs 4 and 5 have not been investigated to determine if they were seed- and pollen-transmitted. Their amplified DNAs were sequenced, and we found they encoded nearly the same amino acid sequence that dsRNA1 encodes (Fig. 4B).

Absence of virus particles

Bands of dsRNAs were slightly detected from even very small amounts (0.025 g) of bark tissue of Japanese pear when gels were stained with ethidium bromide (Fig. 5). The concentration of dsRNAs detected from Japanese pear was nearly constant in any cultivars and any tissues. The concentration of cryptic viruses are also generally known to be constant in any cultivars and any tissues. According to a comparison by electrophoresis, the concentrations of dsRNAs in the host, Japanese pear, were estimated to be about 16 times higher than those of dsRNAs from RYEV, a cryptic virus. We tried to visually identify or purify virus-like particles associated with the dsRNAs. After 2-cycle differential centrifugation, dsRNAs were found in the supernatants, not in the pellets (Fig. 6). The dsRNAs in lanes 2 and 4 were thought to be derived from tiny incompletely-smashed tissues. Viral particles were not found by electron microscopy observation in dip and preparations partially purified by ultracentrifugation, although the dsRNAs concentration in the host, Japanese pear, were considerably high. On the other hand, RYEV particles are found in dip and purified preparations by the same method, in spite of the low concentration. These results suggest that dsRNAs 1, 2, and 3 were not derived from cryptic viruses and were unencapsidated. The dsRNAs in Cryptosporidium parvum, the RNA-dependent RNA polymerase of which had similarity with the deduced protein sequence of dsRNA1, are also unencapsidated.

dsRNAs are not normally found in plants unless they are infected with RNA virus. However, dsRNAs from cryptic viruses and high-molecular weight dsRNAs have been found in various plants without symptoms. The properties of cryptic viruses are: (1) isometric particles, (2) the nucleic acid consists of segmented dsRNA, (3) no

Fig. 4. Northern Dot Blot Hybridization Analysis and Partial Nucleotide Sequence of DsRNA1, 2, 3, 4, and 5 and Deduced Amino Acid Sequence.

(A) DIG-labeled 267-bp product amplified by 1h and 4c from dsRNA1 was used as probe. One twelfth of the dsRNA1 purified from 5 g of bark tissue (approx. 0.5 µg) and half µg of RNA marker were spotted. DsRNA1 (No. 1), dsRNA2 (No. 2), dsRNA3 (No. 3), dsRNA4 (No. 4), dsRNA5 (No. 5) and RNA molecular weight marker III as control (Boehringer Mannheim) (No. 6). (B) The nucleotide sequences of RT-PCR amplified cDNA products from dsRNAs 1, 2, 3, 4, and 5 by primers, 1h and 4c, and amino acid sequences of the coding regions are shown. The conserved amino acid sequence motifs are underlined.

Fig. 5. Comparison of DsRNA Densities in Hosts between DsRNAs 1, 2 and 3 from Japanese Pear and DsRNAs from Cryptic Virus.

(A) The dsRNA preparation loaded was purified from the following fresh weight of Japanese pear cv. Niitaka bark tissue: 25 mg (lane 1), 50 mg (lane 2), 100 mg (lane 3), 200 mg (lane 4), 400 mg (lane 5), 800 mg (lane 6), and 1600 mg (lane 7). (B) The dsRNA preparation loaded was purified from the following fresh weight of radish cv. Minowase leaf tissue: 400 mg (lane 1), 800 mg (lane 2), 1600 mg (lane 3), and 3200 mg (lane 4).
symptoms in their host plants, (4) low concentration in infected plants, (5) high frequency of seed- and pollen-mediated transmission (no transmission via grafting), (6) a constant concentration in their host cells.

The high-molecular weight dsRNAs are not associated with virus-like particles and are further divided into two groups: dsRNAs that are thought to be associated with cytoplasmic male sterility, and endogenous dsRNAs, the origins and biological functions of which 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. Distinct differences between dsRNAs from cryptic viruses and dsRNAs of unknown function are the molecular weight and encapsidation.

Based on the molecular weight, the dsRNAs detected from Japanese pear are similar to dsRNAs of cryptic viruses. But distinct virus-like particles associated with the dsRNAs were not found. The dsRNAs from Japanese pear are similar to high-molecular weight dsRNA the function of which is unknown. In addition, the dsRNAs concentration in the host, Japanese pear, were fairly high. The fact is strange that all deduced amino acid sequences of dsRNAs from Japanese pear, dsRNA1, 2, 3, 4 and 5, contain conserved amino acid sequence motifs found in the genes that encode putative RNA-dependent RNA polymerases of RNA viruses. Their biological functions, mechanisms of replication, and evolutionary relationship to known viruses and plasmids are of considerable interest.

The similarity between plant cryptic viruses and mycoviruses often leads to a hypothesis that the fungus might have acquired ancestral viruses from plant viruses or provided mycoviruses to plant cells through infection. The possibility is also attractive that Japanese pear might have acquired these dsRNAs from plant viruses or mycoviruses of plant pathogenic fungus during fungal infection of Japanese pear, or provided them to plant viruses or mycoviruses.

References

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