Genomic heterogeneity of rice dwarf phytoreovirus field isolates and nucleotide sequences of variants of genome segment 12

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Electrophoretic profiles of the dsRNAs of field isolates of rice dwarf virus (RDV) were compared with those of an isolate maintained at Hokkaido University (RDV-H). Unexpectedly, the genomic dsRNAs of most of the field isolates showed distinct electrophoretic mobility profiles. This was the case even among isolates from the same region. Genome segment 12 (S12) from some variants migrated faster than S12 from RDV-H. These RNAs were converted to full-length cDNAs and sequenced. S12 from all the variants had the same length of 1066 nucleotides with nucleotide sequence identities of 96 to 99%. Three open reading frames previously reported were present in all the variants, and the sequence identities were 95 to 99% for P12, 98 to 100% for P12OPa, and nearly 100% for P12OPb. A comparison of the nucleotide and amino acid sequences of the variants with sequences of the RDV-H and Akita isolate showed that there are two genomic types, one represented by RDV-H and the other by the Akita isolate.

Introduction

Rice dwarf virus (RDV), together with wound tumour virus (WTV) and rice gall dwarf virus, belong to the Phytoreovirus genus of the Reoviridae family (Boccado & Milne, 1984). These viruses all have a segmented genome of twelve dsRNAs. Nucleotide sequence analysis of WTV has indicated that the sequences are highly conserved among the mutants. Defective interfering RNAs derived from WTV segment 5 (S5) had completely identical sequences to those of parental S5 even after 20 years (Anzola et al., 1987). Furthermore, WTV isolated recently from a periwinkle plant (Catharanthus roseus) found in a field in New Jersey, U.S.A. also had S7, S11 and S12 nucleotide sequences very similar to the type of virus maintained for more than 40 years in a greenhouse (Hillman et al., 1991). These studies indicate that the nucleotide sequence of WTV is highly conserved during replication over long periods. In the case of RDV nucleotide sequences determined in different laboratories (S1: Suzuki et al., 1992a; S2: Uyeda et al., 1993; S3: Yamada et al., 1990; Suzuki et al., 1990b; Kano et al., 1990; S5: Suzuki et al., 1990a; Hayashi & Minobe, 1990; S9: Uyeda et al., 1989; Fukumoto et al., 1989; S10: Uyeda et al., 1987; Omura et al., 1988) showed some differences although amino acid sequences are highly conserved.

However, no direct comparison of different RDV isolates was made and the extent of genomic variations has not been studied in detail. This is mainly because no phenotypic variant or strain has been isolated in nature. We collected RDV from fields in different areas of southern Japan and analysed their genomic dsRNAs by using PAGE. The analyses showed that genomic variants were common in nature. The S12 of some variants was further analysed by sequencing.

Methods

Direct extraction of genomic dsRNAs. Infected leaves were homogenized in 5 to 10 vol. of STE buffer (10 mm-Tris-HCl pH 6.8, 0.1 M-NaCl, 1 mM-EDTA) containing 1% SDS, 1% 2-mercaptoethanol and 0.5% bentonite, and then extracted twice with an equal volume of phenol:chloroform (chloroform:isoamyl alcohol 24:1) 1:1. Nucleic acids in the aqueous phase were precipitated using ethanol, and ssRNAs were excluded by 2 M-LiCl precipitation (Diaz-Ruz & Kaper, 1978). Genomic dsRNAs were further purified with microgranular cellulose CC41 (Whatman) as described by Dufle & Bar-Joseph (1989).

PAGE. For comparison of genomic dsRNA migration patterns, 10% polyacrylamide gels (Laemmli, 1970) were used. Separating gels were prepared using a degassed solution of 10% acrylamide (30% acrylamide and 0.8% N,N'-methylene-bis-acrylamide), 0.375 M-Tris-HCl (pH 8.9) and 0.1% SDS containing 0.1% N,N',N',N'-tetramethylethylenediamine (TEMED) and 0.1% ammonium sulphate, and poured into a 16 × 16 × 0.1 cm slab gel apparatus. To obtain sharp bands, stacking gels of 3% acrylamide, 0.0625 M-Tris-HCl pH 6.7 and 0.1% SDS were laid on the separating gels. Electrophoresis was performed for 20 h at 100 V using a solution of 0.05 M-Tris, 0.05 M-glycine and 0.1% SDS as electrode buffer and the gels were stained in 0.5 μg/ml ethidium bromide. The bands were visualized under u.v. light.

Northern hybridization. The extracted dsRNAs were loaded on 5% polyacrylamide gels in TAE buffer (Sambrook et al., 1989) and
transferred to a nylon membrane (Hybond-N+, Amersham) in 0.2 M-NaOH for 45 min at room temperature. These transferred RNAs were then hybridized with 5' terminally labelled genomic dsRNAs of RDV-H. Prehybridization was performed at 42 °C for 2 to 3 h in 50% formamide, 5× SSC, 50 mM-sodium phosphate buffer pH 6.7, 500 µg/ml salmon sperm DNA, 0.1% SDS and 5× Denhardt’s solution. Hybridization was then done at 52 °C overnight.

cDNA cloning. The cDNAs to RDV-H were made from transcripts (Uyeda & Shikata, 1984) using the method of Gubler & Hoffman (1983). After homopolymeric tailing of the cDNAs, they were inserted into oligo(dG)-tailed pBR322 at the PstI site. The cDNA clone pRD1A1 was selected and a probe made from the cDNA insert reacted specifically with S12 in a dot-blot hybridization. cDNA clones from RDV-H were also made from genomic RNAs by reverse transcription coupled with a PCR (RT-PCR). Genomic dsRNAs were extracted, denatured with 90% DMSO and reverse-transcribed by avian myeloblastosis virus reverse transcriptase XL using a primer complementary to 15 nucleotides at the 3' terminus. After phenol extraction and ethanol precipitation, the first strand cDNA was subjected to PCR. The 50 µl reaction mixture contained 10 mM-Tris•HCl pH 8.9, 1.5 mM-MgCl₂, 80 mM-KCl, 25 µg BSA, 0.1% sodium cholate, 0.1% Triton X-100, 200 µM-dNTP, 1 µg oligonucleotide primers complementary to the 5' and 3' termini, and 4 units of Tth DNA polymerase. The full-length cDNAs were amplified by denaturing them at 94 °C for 1 min, annealing the primers at 55 °C for 2 min, and then performing extension at 72 °C for 3 min for 25 cycles in a temperature-controlled ASTEC PC-700 (Astec). cDNAs were subcloned into pUC119 or Bluescript II SK-.

Full-length cDNAs to the S12 of field-collected RDV variants were obtained by RT-PCR of the denatured genomic dsRNAs (2 to 7 µg) using 5' (5' ccctgeaGGTAAATTGAGCAGTATTT 3') and 3' end (5' gcctcagtACATCGTATGACG 3') primers deduced from the sequence of RDV-H. Restriction enzyme sites (underlined) for PstI and XbaI were added to the 5' termini of the 5' and 3' end primers, respectively. The PCR products were cloned into plasmid pUC119.

Sequencing. Sequencing of the cDNA was performed by the dideoxynucleotide chain termination method (Sanger et al., 1977) using a Sequenase version 2.0 DNA sequencing kit (United States Biochemical). Deduced nucleotide sequences were assembled and analysed using the computer program SDC-GENETYX (Software Developing Co.).

Results

Differentiation of RDV isolates by electrophoresis of dsRNAs

Infected plants were collected from nine locations (Table 1). When the extracted dsRNAs were compared using 10% PAGE, the migration patterns differed among most samples, and isolates could be differentiated by using these patterns. Fig. 1 shows a PAGE comparison of isolates from these nine locations. Unexpectedly, migration patterns differed even among isolates collected from a single location (data not shown). Only two pairs among the nine samples from Niita revealed the same migration pattern. A similar variation within a single location was observed in isolates W, AK, KA, KA and KA.

In addition, heterogeneous bands were detected at S7

Table 1. Rice dwarf virus isolates

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Fig. 1. Comparison of the genomic dsRNA migration profiles of RDV variants obtained from different geographical areas. The dsRNAs directly ex from rice leaves were loaded on 10% gels and stained with ethidium bromide. The isolates used are indicated above by abbreviations (see Table 1). Segments are numbered on the left. Lanes 1 to 3, 4 to 8 and 9 to 14 represent three separate gels, but RDV-H was included as a standard in each gel.
Fig. 2. Sequence relatedness of RDV variants. The dsRNAs of five isolates (see Table 1) were directly extracted from rice leaves, loaded on 5% gels and stained with ethidium bromide (a). The bands were transferred to a nylon membrane and hybridized with 5' terminally labelled dsRNAs of RDV-H (b). The isolates used are indicated above and the segments are numbered in the centre.

and S9 of RDV-W, S12 of RDV-AN and S6 of RDV-K (Fig. 1). The staining intensities of these double bands were weaker than those of single bands indicating that these isolates do not have additional genome segments but that at least two variants infected the same plant or mutations of these segments occurred in the infected plant.

Sequence relatedness between different genomic variants

The sequence relatedness between genomic variants was examined by Northern blotting using ^32P-labelled total genomic dsRNAs of RDV-H as probes (Fig. 2). All the bands detected in 5% PAGE (Fig. 2a) hybridized with the probe showing that none of the segments were unique to the variants tested.

Nucleotide sequence of RDV-H

The clone pRD1A1 had a cDNA insert of 815 nucleotides and contained a complete 5' terminus since the sequence following the oligo(dG) tail was identical to that obtained by direct RNA sequencing as reported by Kudo et al. (1991). Because the clone did not contain the 3'-terminal region, a full-length cDNA was made by PCR after first-strand cDNA synthesis from the genomic dsRNA. cDNA was made using a primer (5' CCATC-GATCAGTTATGAGCAG 3'), which is complementary to the 3' terminus. The cDNA was then amplified using this primer and an additional primer (5' CCCTGGAGTAATATTGAGCAGTATTT 3'), complementary to the 5' terminus. The amplified full-length cDNAs were then subcloned into the poly linker region of Bluescript II SK- and sequenced. The complete nucleotide sequence is presented in Fig. 3. It is 1066 nucleotides long, with the longest open reading frame starting at nucleotide 42 and terminating at nucleotide 978.

cDNA cloning of S12 of RDV variants

When all the variants were subjected to RT-PCR and analysed by 1.0% agarose gel electrophoresis, one major band of the expected size was detected. After insertion of the cDNAs into a plasmid vector, three clones were used
for nucleotide sequencing. To examine the reliability of the sequence data from PCR-generated clones, the copy number of first-strand cDNA generated by reverse transcription was estimated by comparing this value with those obtained using full-length cDNA as a template (Fig. 4). Based on the band intensity of the amplified DNA in the agarose gel, the copy number of first strand cDNA was estimated to be $2 \times 10^6$ to $2 \times 10^8$. Therefore it is unlikely that cDNA clones randomly selected were from the same template used during the PCR cycle. Sequences were determined on the basis of at least two clones being found to have the same nucleotide. There were no positions in the nucleotide sequence where all three clones had different bases.

### Comparison of nucleotide sequences of S12 of RDV variants

To study the relationship between mobilities in 10% PAGE and differences at the genomic structural level, S12 sequences were obtained. Fig. 1 shows that S12 could be separated into two groups, namely those with slow mobility [RDV-II, AK and AN (upper band)], and those with fast mobility [RDV-N, W, AI, AN (lower band), K, KaN, KaK and KaM]. From among these isolates, S12 sequences of RDV-N, W and AK were determined. Three cDNA clones were sequenced for each isolate. First, 240 bases of the 5' termini and 330 bases of the 3' termini were analysed using universal primers for sense RNA and 154RD 12B is complementary to nucleotides 821 to 837 of positive-sense RNA. The results were compared with those for RDV-H and the RDV Akita isolate (RDV-Akita) (Suzuki et al., 1992b) (Fig. 3). All S12 sequences of these variants were 1066 nucleotides long. No deletions or insertions were found, and only substitutions were detected. Nucleotide sequence identi-
Table 2. Differences in deduced amino acid sequences*

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* Amino acids are represented in single-letter code. Only amino acids differing from those of RDV-H are shown, and spaces indicate identity with RDV-H.
† Open reading frame (ORF) P12OPb is not shown because it is in-frame within P12OPa (Fig. 3).
‡ Positions are numbered from the N terminus of each polypeptide.

Differences were 96 to 99%. Fig. 3 shows that variants of S12 could be separated into two types by their sequence homology i.e. RDV-H and -AK as type 1 and RDV-N, -W and -Akita as type 2. This result was consistent with the groupings based on mobility in 10% PAGE. Because for RDV-AN two bands were seen in 10% PAGE (Fig. 1), cDNAs to S12 of RDV-AN were prepared by RT-PCR without separation of these two bands and then sequenced. Two sequences were obtained (results not shown); one was classified as belonging to the RDV-H and -AK group whereas the other was type 2. Therefore, it was thought that the former and latter corresponded respectively to the upper and lower S12 bands of RDV-AN obtained by 10% PAGE.

Comparison of deduced amino acid sequences from S12 nucleotide sequences

Amino acid sequences encoded by S12 were deduced from nucleotide sequences and compared (Table 2). Three open reading frames reported by Suzuki et al. (1992b), P12 (312 amino acids), P12OPa (92 amino acids) and P12OPb (84 amino acids), were present in all variants (Fig. 3). Sequence identities of the deduced amino acid sequences were 95 to 99% for P12 and 98 to 100% for P12OPa; P12OPb was highly conserved among the variants and had nearly 100% identity.

Discussion

The presence of genomic variants of RDV has been reported only once before; the variant originated by a spontaneous mutation (Kimura et al., 1987). However, the present study shows that genomic variants are common in the southern part of Japan. Most isolates differed from one another in at least one of the 12 segments, even when derived from the same field. This could be due to a reassortment of the corresponding genomic segments. The presence of double bands at the position of certain genome segments, S7 and S9 of W (Fig. 1 lane 3), S12 of AN (Fig. 1 lane 6) and S6 of K (Fig. 1 lane 9), indicates that mixed infection by variants can occur in a single plant. Indeed, segregation of these heterogeneous segments can be experimentally achieved by transferring the virus through an insect vector (I. Uyeda, unpublished).

Nucleotide sequence analysis of S12 variants showed that they fall into two types. These types did not correlate with the geographical areas because one isolate...
contained both types (Fig. 1 lane 6). An examination of whether there are also two types in other genomic segments would be of interest in terms of viral evolution and reassortment.

Recent studies have concentrated on determining the genomic structure of RDV. Although the genome segments have been sequenced, little has been revealed as to their biological function. In this study, variants distinguishable by the migration patterns of their dsRNAs in PAGE were obtained, and these could be useful for determining gene functions, as has been done for rotaviruses (Estes & Choen, 1989; Ramig & Ward, 1991).

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References


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