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CHARACTERIZATION OF THE cDNA CLONE TO RICE BLACK-STREAKED DWARF VIRUS GENOME SEGMENT 10.

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I. Introduction

Rice black-streaked dwarf virus (RBSDV) belongs to the plant reovirus subgroup 2. Viruses in the subgroup 2 are transmitted by planthoppers and are a spherical particle with a distinct double shell in diameter of about 70 nm (8). The viruses have 10 segmented dsRNAs as a genome and have virion-associated RNA polymerase activity (17). In order to elucidate mechanisms operating in gene expression, replication, and morphogenesis of a virus, it is essential to characterize the genome by cloning its cDNA into bacterial plasmids. Molecular cloning of genome segments of plant reoviruses have been reported for wound tumor virus (WTV) (1, 2, 3, 4), rice dwarf virus (RDV) (12, 16, 18), and rice ragged stunt virus (RRSV) (9). And complete nucleotide sequences of the several segments of WTV and RDV has been determined. The sequence analyses of the genome segments showed that all genome segments of WTV and genome segments 9 and 10 of RDV had the common conserved terminal sequence of 5'GGUA — U/CGAU3'. Since the terminal sequences of the genome segments are expected to play an important role in replication of the viral genome as pointed out in ssRNA viruses (19), the common sequence indicated the common ancestral origin of the two viruses. But none of the genome segments of the viruses in the subgroup 2 has been cloned nor sequenced. In this communication, molecular cloning of the genome segment 10 was presented and nucleotide sequences of its terminal portions were determined.

II. Materials and Methods

Genome dsRNA extraction. The virus was purified from infected maize leaves and sheath as previously described (17). Genome dsRNA was extracted from the purified subviral particles by the method of Uyeda and Shikata (15).

The individual dsRNA segments were isolated by 5% polyacrylamide gel electrophoresis in 40 mM Tris, 195 mM sodium acetate and 2 mM EDTA.

Preparation of viral transcript. Transcription reactions contained 100 mM Tris-HCl (pH 9.0), 4 mM MgCl₂, 4 mM phosphoenolpyruvate, pyruvate kinase (2.5 mg/ml), 1 mM each of GTP, ATP, CTP and UTP, 1 mM S-adenosyl methionine, 5% bentonite, purified subviral particles and were incubated for 15-17 hr at 30°C (17). Synthesized transcript was isolated by 2 M LiCl treatment (15).

cDNA synthesis and cloning. Mixture of all genome segments were denatured in 40% DMSO at 95°C for 5 min and the 3' termini of both strands of the denatured RNA were polyadenylated using poly A polymerase (Takara shuzou). cDNA copies of polyadenylated RNA were synthesized using reverse transcriptase and oligo [dT] as a primer. RNA was then degraded in 0.3 M NaOH at 37°C and neutralized with HCl. cDNAs were then passed through Bio gel-A15 m column (6 mm×22 cm). cDNAs of opposite polarities were annealed and protruding termini of the annealed cDNA was filled using Klenow fragment (Takara shuzou). And it was inserted into pBR 322 at *Pst* I site after homopolymeric tailing. And *E. coli* strain HB 101 was transformed with the recombinant DNAs according to the CaCl₂ method described by Maniatis *et al* (11).

Agarose gel electrophoresis. The size of the cDNA inserts was estimated by 1% agarose gel electrophoresis. Plasmids were prepared by the alkaline lysis method described by Maniatis *et al* (11).

Dot blot hybridization. Gel-purified individual genome segment (40 ng) was denatured at 100°C for 10 min, quenched on ice, and spotted onto nitrocellulose paper presoaked in 20×SSC with an aid of a Hybri-Dot manifold (Bio rad). The spotted membrane was air-dried and baked for 2 hr at 80°C. Prehybridization was performed at 42°C over night, while hybridization was performed at 42°C over night with 1×10⁶ cpm of heat-denatured and ³²P-labeled cDNAs in a volume of 1 ml. Plasmids with cDNA inserts were labeled by fill-in reaction using [α -³²P] dCTP and Klenow fragment after digestion with restriction enzyme *Hpa* II. Hybridization and prehybridization solutions were consisted of 5×SSC, 0.1% SDS, 5×denhardt's solution (11), 0.05 M sodium phosphate (pH 6.8) and 50% formamide. Following hybridization, the dot-blots were washed four times for 15 min each with 2×SSC-0.2% SDS and autoradiographed.

Primer extension. Digestion with *Hin* dIII and *Pst* I yielded fragments of about 400 nucleotides from both 5' and 3' termini. These fragments were further digested with *Dra* I and *Sau* 3A, and *Alu* I and *Sau* 3A to yield primer 1 and 2, respectively. The restriction fragments were isolated from 10% polyacrylamide gel containing 8 M urea after labelling 5' termini with [γ -³²P]ATP and denaturation at 95°C for 5 min. And the fragments were eluted in elution buffer (0.5 M ammonium acetate, 1 mM EDTA, 0.1% SDS). The heat denatured genome RNA and 5'-³²P-labeled restriction fragments were annealed in a sealed capillary tube in

0.3 M KCl, 50 mM Tris-HCl (pH 8.0) by heating at 95°C for 5 min and cooling slowly to 42°C. The annealed primers were extended by reverse transcriptase in 250 mM Tris, pH 8.0, 50 mM MgCl₂, 50 mM Dithiothreitol and 1 mM each of ATP, GTP, UTP and CTP.

Direct RNA sequencing. Synthetic oligonucleotides I and II were designed based on the cDNA sequence that are complementary to about 50 nucleotides downstream from the termini and used as a primer. Primers was isolated by 17.28% polyacrylamide gel containing 8 M urea, and eluted in an elution buffer after 5'-³²P-labelling. Sequencing condition was the same as described above, but the reaction mixture contained ddNTP (the ratio of ddNTP to dNTP was 1 : 4 to 1 : 1).

III. Results and Discussion

Characterization of the recombinant clone pRB C3.

Plasmid pRB C3 contained the largest insert among 46 transformants generated from 13 µg genome dsRNA. The insert of the pRB C3 was about 1800 base pair (Fig. 1) and was further characterized. In dot blot hybridization, the ³²P-labeled plasmid reacted specifically only to the genome segment 10 (Fig. 2).

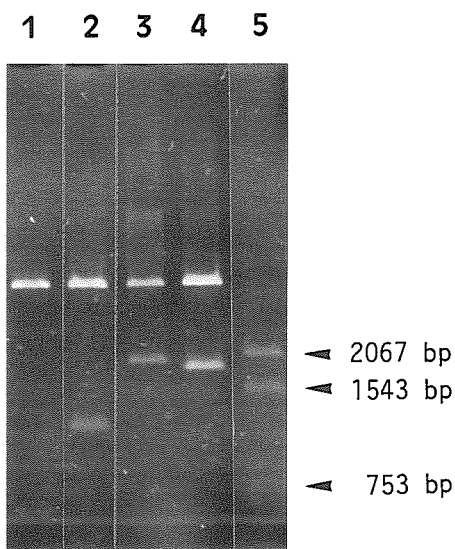


Fig. 1. Sizing of cDNA inserts by 1% agarose gel electrophoresis. Lanes 1 to 4 show profiles of recombinant plasmids after *Pst* I digestion. Lanes 1, 2, 3 and 4 are pRB D5, pRB A3, pRB C2 and pRB C3, respectively. Lane 5 is size markers.

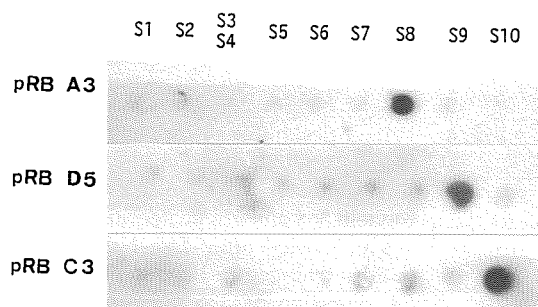


Fig. 2. Assignment of cDNA clones to genome segments of rice black-streaked dwarf virus by dot blot hybridization. Genome segments (S1-S10) separated by polyacrylamide gel electrophoresis were spotted onto a nitrocellulose sheet and hybridized with ³²P-labeled plasmids pRB A3, D5 and C3.

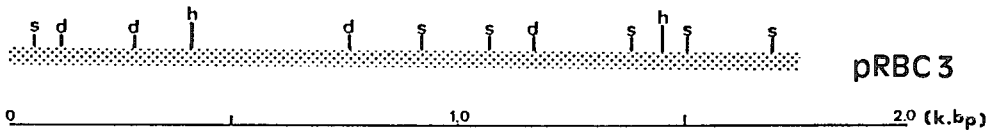


Fig. 3. A restriction enzyme cleavage map of cDNA insert of pRB C3. s : *Sau* 3A. d : *Dra* I. h : *Hin* dIII.

A restriction enzyme cleavage map of the pRB C3 is presented in Fig. 3. Since the molecular weight of the genome segment 10 was 1.06×10^6 , which is calculated to be about 1600 nucleotides, the plasmid pRB C3 was considered to cover the near full-length of the genome segment 10.

In order to determine whether this clone was a complete cDNA of the segment, primer extension analyses were done using restriction endonuclease cleavage fragments of both terminal portions of the cDNA insert as a primer. *Hin* dIII fragments of the cDNA clone was sequenced and primers were designed based on this sequence (Fig. 4). Primer 1 was a 46 nucleotides of *Dra* I and *Sau* 3A fragment and Primer 2 was a 51 nucleotides of *Alu* I and *Sau* 3A fragment. Using genome dsRNA denatured in DMSO as a template, the primer 1 terminated at a position of 102 nucleotides and the primer 2 did at positions of 60 and 61

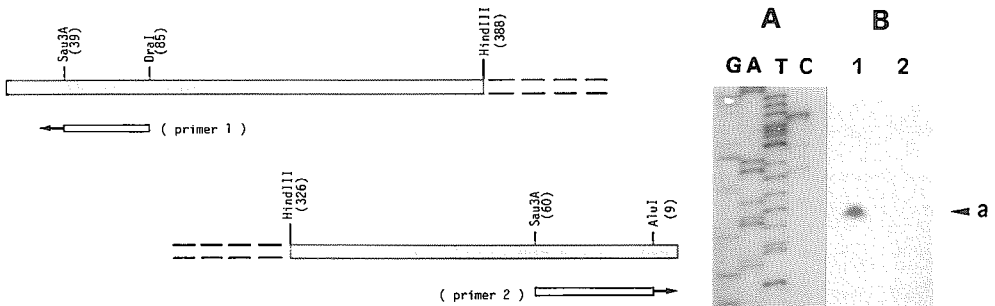
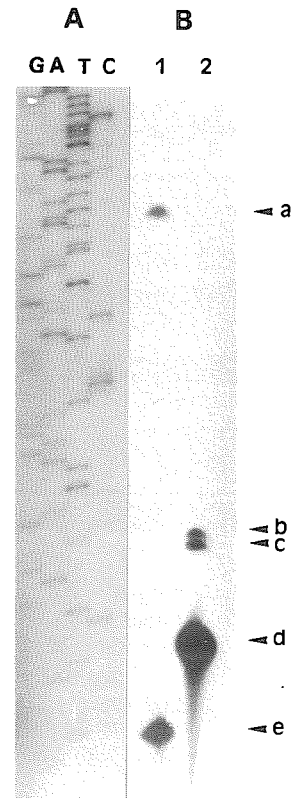


Fig. 4 Primer design for primer extension analyses of rice black-streaked dwarf virus genome segment 10. pRB C3 insert was digested with *Pst* I and *Hin* dIII to yield 388 and 326 nucleotides fragments. Primer 1 was labeled after *Dra* I digestions of 388 nucleotides fragment. Primer 2 was labeled after *Sau* 3A digestion of 326 nucleotides fragment. After the labelling, the fragments was digested with *Sau* 3A and *Alu* I to yield primer 1 and 2, respectively.

Fig. 5. Mapping of 5' and 3' termini of RBSDV genome segment 10. A : Sequence ladder of M13 mp18. B : Primer extension analyses. The positions a, b, and shows terminated positions of 102, 60 and 61 nucleotides, respectively. d and e are primer 2 and 1, respectively.



nucleotides (Fig. 5). This showed the cDNA was 16 nucleotides short from one terminus and covered entire nucleotides of the other terminus. The second termination bands using the primer 2 may be an artifact of reverse transcription.

Terminal nucleotide sequence of the genome segment 10.

Terminal nucleotide sequence of the genome segment 10 was determined by sequencing both viral RNA and the cDNA. For RNA sequencing, synthetic

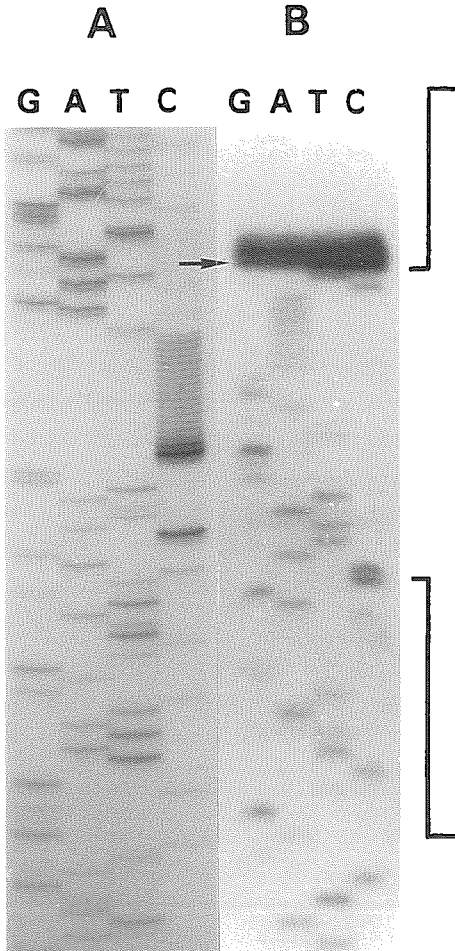


Fig. 6. 5' terminal nucleotide sequence of rice black streaked-dwarf virus genome segment 10 analyzed by pRB C3 (A) and viral transcript (B). The oligo[dC] track of panel A was added to cDNA during molecular cloning. An arrow shows a site of termination.

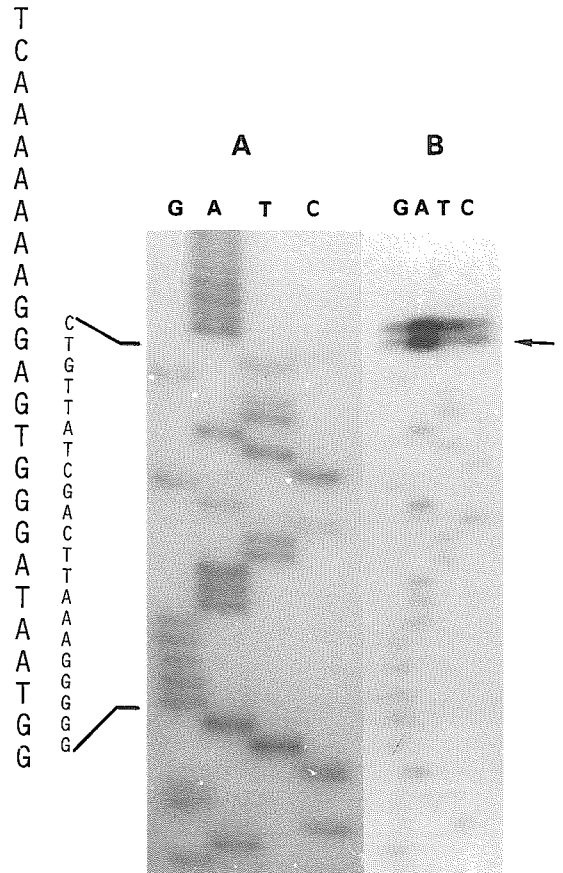


Fig. 7. 3' terminal nucleotide sequence of rice black streaked-dwarf virus genome segment 10 analyzed by pRB C3 (A) and viral genome dsRNA (B). The oligo[dA] track of panel A was added to cDNA during molecular cloning. An arrow shows a site of termination.

oligonucleotides I and II were designed based on the cDNA sequences that are complementary to about 50 nucleotides downstream from the termini and used as a primer. Since the oligonucleotide I was active as a primer when viral transcript was used as a template, the sequence obtained by this primer was concluded to be that of 5' terminus of plus sense (Fig. 6). The sequence obtained using the oligonucleotide II was identical to that of cDNA followed by oligo [dA] (Fig. 7).

The sequence 5'NAGUUU — UGUC3' of the genome segment 10 of RBSDV was found to be different from the conserved terminal sequence of WTV and RDV. Thus the presumptive terminal conserved sequence of RBSDV, a member of the plant reovirus subgroup 2, was at least different from that of viruses in the plant reovirus subgroup 1. The determination of the conserved terminal sequences of the genome segments of RBSDV and other viruses in the plant reovirus subgroup 2 will give some insights in terms of the origin of plant reoviruses.

IV. Summary

A cDNA clone to rice black-streaked dwarf virus (RBSDV) genome segment 10 was made and characterized. The clone pRBC3 contained a cDNA insert of about 1800 nucleotides and specifically hybridized to the genome segment 10 by a dot-blot hybridization. A restriction endonuclease cleavage map of the cDNA clone was constructed. Primer extension analyses using restriction fragments as a primer and genomic RNA as a template indicated that the clone covered a near full-length of the genome segment 10. The genome segment had the terminal structure of 5'NAGUUUUUUU — AUUGUC3'. Thus presumptive conserved terminal sequence of RBSDV was different from those of rice dwarf virus.

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