

イネラギッドスタントウイルス(RRSV)ゲノムセグメント10 のクローニングと全塩基配列

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Molecular Cloning and Complete Nucleotide Sequence of Rice Ragged Stunt *Oryzavirus* Genome Segment 10

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Abstract

cDNAs to rice ragged stunt *Oryzavirus* genome segment 10 (S10) were made by oligo-dT primed 1st strand cDNA synthesis after poly A addition to 3' terminal of viral transcripts and cloned in a bacterial plasmid pBR322. The overlapping cDNA clones were sequenced and the assembled sequence covered the full-length of the genome except for 22 nucleotides from the 5' terminus, which were determined by a direct RNA sequencing method. RRSV S10 is 1162 nucleotide long and an inverted repeat structure containing 6 nucleotides was found in the terminal region. It has a mini ORF in addition to the downstream long ORF. The upstream mini ORF encodes 12 amino acids. Presence of this mini ORF was confirmed by sequencing another full-length cDNA clones generated by reverse transcription coupled with a polymerase chain reaction. The long ORF encodes a polypeptide of 297 amino acids (M_r 32.4 K).

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Key words : rice ragged stunt *Oryzavirus*, genome segment 10, complete nucleotide sequence.

INTRODUCTION

Rice ragged stunt disease was first observed in 1976-1977 in Indonesia and the Philippines^{7,11}. Rice ragged stunt *Oryzavirus* (RRSV) has an icosahedral particle of approximately 65 nm in diameter, possesses a genome consisting of 10 double-stranded (ds) RNA segments and has been shown to be a member of the family Reoviridae^{2,8,14}.

Nucleotide sequence information is an essential requirement to examine the inter-viral relationship and to analyze gene functions at molecular level. To date, the complete nucleotide sequence has been determined for genomic segments of some phyto-reoviruses and Fujiviruses. Among phyto-reoviruses, nucleotide sequences of all the genome segments has been determined for rice dwarf virus (RDV) and relatedness between the phyto-reoviruses were well documented²⁰. Nucleotide sequence elements potentially involved in viral RNA sorting and packaging events has been proposed for wound tumor virus (WTV)¹. The assignment of structural and non-structural viral polypeptides to most of the genome segments has also been made for RDV and WTV^{12,16,18,20} based on the sequence information. In contrast, very little sequence information, only the conserved terminal sequences^{23,24} of RRSV and Echinochloa ragged stunt virus and the complete nucleotide sequence of RRSV genome segment 9 (S9)²², have been reported for

oryzaviruses.

In this study, molecular cloning of RRSV S10 was performed and its complete sequence was determined.

MATERIALS AND METHODS

cDNA synthesis. Panel of cDNAs were synthesized by a Gubler and Hoffman's method⁴) as described by Lee *et al.*¹⁰) or using a cDNA synthesis kit (Amersham International plc., USA).

The polymerase chain reaction (PCR) method was used to synthesize the full-length cDNAs to RRSV S10. After denaturation of the total genomic dsRNAs with DMSO, the first strand cDNA to RRSV S10 was synthesized in the reaction mixture of 50 μ l containing 50 mM Tris-HCl, pH 8.0, 50 mM KCl, 4 mM DTT, 8 mM MgCl₂, 2.5 μ g actinomycin D, 1 mM dNTPs, 2.5 μ g genome RNA, and about 10 units of reverse transcriptase XL (Takara Shuzo Co., Ltd.). One μ g of synthetic oligonucleotides (22 mer) designed based on the 3' terminal sequence of RRSV S10 was used as a primer. The mixture were incubated at 48°C for 1 hr. The first strand cDNAs were extracted from reaction mixture with phenol/chloroform, precipitated with ethanol, and resuspended in 25 μ l H₂O. For PCR, primers (22 mer) complementary to 5' and 3' terminal sequences of S10 were used. The reaction mixture of 50 μ l consists of 5 μ l of reaction buffer (15 mM MgCl₂, Toyobo Co. Ltd.), 100 μ M dNTPs, 0.5 μ g 5' and 3' primers, 1 μ l of first strand

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cDNA and 2.5 units thermostable DNA polymerase (Tth DNA polymerase, Toyobo Co. Ltd.). The PCR reaction was carried out by 25 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 2 min, primer extension at 72°C for 3 min. Then, amplified cDNAs were extracted by phenol/chloroform and precipitated by ethanol. The cDNAs were cloned into a plasmid pUC119 and sequenced.

Assignment of cDNA clones to S10. Assignment of the cDNA inserts to S10 was made by dot-blot hybridization as in Lee *et al.*¹⁰⁾, using ³²P-labeled S10 RNA as a probe.

The identification of cDNA clones covering 3' terminus was made by a colony hybridization. Colonies were simultaneously consolidated onto a master agar plate and onto Hybond N⁺ membrane (Amersham International plc.) laid on the surface of a second agar plate. After an appropriate period of growth, the membrane was laid, colony face up, on Whatman 3MM paper moistured with 2×SSC containing 5% SDS and left for 3 min. The dish with the membrane was then transferred onto a rotating turntable in a microwave oven (Sanyo, Model EM-M530T) and treated for 1 min at full setting (960 watts). Then the membrane was wetted in 5×SSC containing 0.1% SDS, washed 2–3 times in 2×SSC and dried at room temperature for 30–60 min. The membrane was stored under a vacuum at the room temperature before using in the hybridization. Synthetic oligonucleotides complementary to the 3' terminal 12 nucleotides was used as a probe. Prehybridization was performed at 50°C for about 3 hr containing 4 ml of 5×SSC, 5× of Denhard's solution, 0.05 M sodium phosphate, pH 6.8, 0.1% SDS and 0.1 mg/ml yeast tRNA. Hybridization was carried out at the temperature of 5°C below the *T_m* (33°C) for about 20 hr with 2.0×10⁵ cpm of

probe in a volume of 1 ml. Following the hybridization, the membrane was washed four times for 10 min each with 2×SSC–0.1%SDS and once for 20 min with 0.1×SSC containing 0.1%SDS.

Sequence analyses.

1) cDNA sequencing Sequence reactions were carried out by the dideoxynucleotide chain-termination method¹⁹⁾ using Klenow fragment (7-DEAZA sequence kit, Takara Shuzo Co. Ltd., Japan) or T7 DNA polymerase (Sequenase version 2.0 kit, United States Biochemical Corporation, USA).

2) Direct RNA sequencing The viral transcript RNA and genome RNA were sequenced directly by reverse transcription method¹⁹⁾.

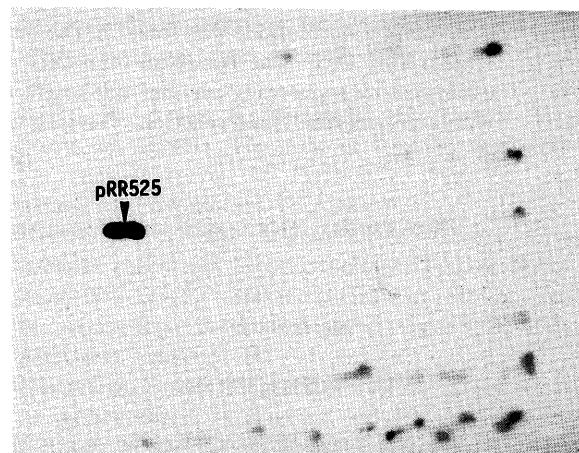


Fig. 2. Identification of cDNA clones to the 3' terminal region of RRSV S10 by a colony hybridization. pRR525 shows a positive autoradiographic signal.

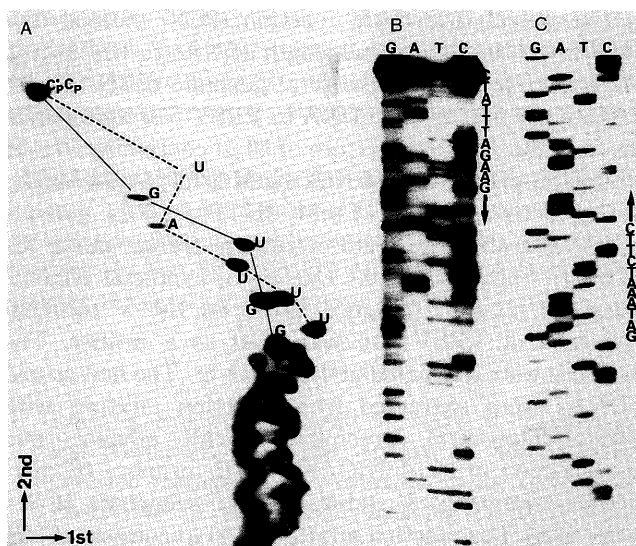


Fig. 1. 5' terminal nucleotide sequence of RRSV S10 obtained by the wandering spot method (A) (Data from Yan *et al.*²³⁾), directly from viral transcripts using reverse transcriptase (B), and from the full-length cDNA (C).

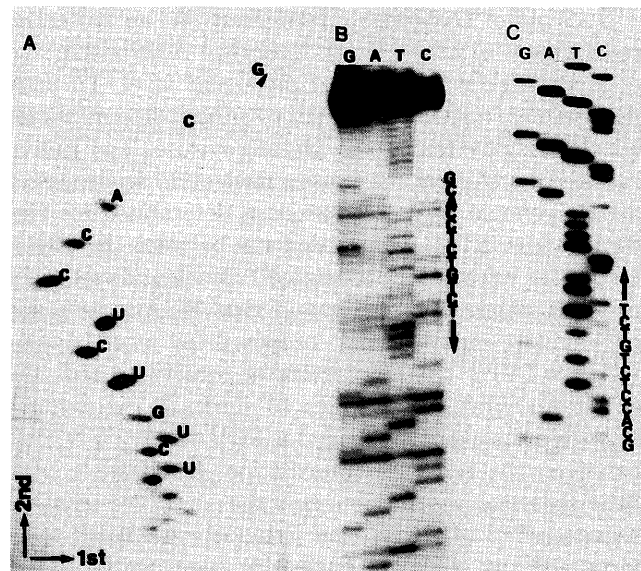


Fig. 3. 3' terminal nucleotide sequence of RRSV S10 obtained by the wandering spot method (A) (Data from Yan *et al.*²³⁾), and from cDNA clone pRR525 (B) and the full-length cDNA (C).

RESULTS AND DISCUSSION

Characterization of cDNA clone pRR124

Among five recombinant DNAs positively hybridized with S10 genomic dsRNA probe out of about 1000 transformants (Data not shown), plasmid pRR124 had the largest insert and was used for sequence analysis.

For sequence analysis, cDNA of pRR124 was subcloned into M13 phage and a series of sequential deletions of the cDNA was made by the method of Henikoff⁶⁾. The size analyses of the deleted cDNA clones was made by digesting with restriction enzyme *Dra*I, and electrophoresing in 2% agarose gel with 2× TBE buffer. A deletion series containing different sizes of cDNAs was sequenced. The *Hind*III site at nucleotide position 571 in pRR124 was also used to cleave and subclone the two different fragments into M13 vectors.

Two subclones (124HPO₃ and 124HPO₅) were obtained and sequenced. The sequences obtained were identical to that read from the deletion clones. From the subclones of plasmid pRR124, about 840 bp of S10 were determined.

The sequence in pRR124 does not contain either of the terminal sequences 5' GAUAAA...GUGC 3' that was found in dsRNAs of RRSV by the wandering spot method²³⁾.

Nucleotide sequence in 5' terminal region

In order to determine the nucleotide sequence of the 5' terminal region, the viral transcripts and genome RNAs were sequenced directly using reverse transcriptase.

The results of RNA primer sequencing are presented in Fig. 1-B. Both transcripts and genome contained the 5' terminal sequence identical to that determined²³⁾ earlier (Fig. 1-A). It was found that plasmid pRR124 lacked 22 bp nucleotides from the 5' terminus.

GATAAATCTTCCGAGCTAAATGCCAATAAGCAGGTTGACACCCGAAACCCCGCATAGCT	60
N P I S R L T P E T P A *	
GTCTGAGGAGTTCTAGTTTCCACCCCAATCGATTCAACGTGACCAATCAATCTTCTCAA	120
TCAATATCAAAACCATCAATCATGCCTTTCGTGCAATTCGGAACCTTGTTTGAAATCAGT	180
M P F V Q F P N L F E I S	
AAATTCGCAAGACAAGGCAAGACTGTTTCTGAATTGAAATGTGAAGTTTGGACTGATTTG	240
K F A R Q G K T V S E L K C E V W T D L	
CTATCATATCTTCTGACTGGATTGCCAAGCTGATTGTTGTCTGATTTTGTGAGCATCAT	300
L S Y L R T G L P T G L L S D F A E H H	
GAACTTAATCAATTGCAAGCGTTACAGCCGTTGATTTGATGAACCATGTTTCGTTCTA	360
E L N Q L Q A F T A V Q F D E P C F V L	
CCAGCCCGAGCCGCATCATAGTATACTGCTGAGCAAGATGATATGCTGTCTGGAGTT	420
P A R A A I I V Y C P E Q D D M L S G V	
TTTGAGGTTGACGCCACAGGTAAGCGTACATTCGTTAGAACGTCAAACACTGATATCATC	480
F E V D A T G K R T F V R T S N T D I I	
GGTTTCAGCAAAGAGTGACGTAAGTGGTGAAAAACAGATTCAATCAGTCGGTGTAGCACAA	540
G S A K S D V S G G K Q I Q S V G V A Q	
GGACTTGAACTGTGATGCAAAATGATGGATTATATTCTGATTCAATTTTCATGTACAATTT	600
G L E T V M Q M M D Y I L I Q F H V Q F	
GGAAGCTTTACAGACATCGGACATTTTGGGATGATGCGGATGCTATCCAACCTGTATGGA	660
G S F T D I G H F G M M R D A I Q L Y G	
ACATGCGCCTGTCCCTTCTGCTTAGCCCTAGCCCGTTTCTCCACTGCGCTGTCTTATCTG	720
T C A C P F L L S L A R F S T A L S Y L	
AATGCCAAGTTGCCATCTATCGTAGGACTGCATTACAGTGGTGAACCAACTACTCTTGGA	780
N A K L P S I V G L H Y S G E P T T L G	
GGCATCATCACTCGTGGTGTAAATTTGAGCGCTCGCGAAGCCTACTTTTCAAAGAAATAT	840
G I I T R G V N L S A R E A Y F S K K Y	
GATCCTGCGCAATCCTTGGTGTGAGTGCTTTCTTTACAGTTAAGACGTCTGCGAGCGGA	900
D P A Q S L V V S A F F T V K T S A S G	
GCTACGGTGATTGAGAAGATGTCAAGCGACATTGGTCTGGTTTATCATATGAACCGCGCT	960
A T V I E K M S S D I G L V Y H M N R A	
GCTGCAGTTAAGGTCTGCTAGCTCTAGAATTGGAAGACTTGGCGAGGTGGCTAACTTTGGT	1020
A A V K V V S S R I G R L G E V A N F G	
GATGACGCAGAGTAGTTCTTCCACTAGGAAGGTTGAGCGGAGCTGAGATGGTAAGGGTG	1080
D D A E *	
GCAGAATGAGACCAGGGGATACGCGGGGGAAGCTGATCTCGATTAGCTAGGGCTAGGCT	1140
GAAAAGGAAGAGACAGAGGTGC	1162

Fig. 4. cDNA nucleotide and predicted amino acid sequences of RRSV S10. The asterisks(*) indicate positions of termination codons.

Nucleotide sequence in 3' terminal region

cDNA of the 3' terminal region of S10, which was not covered by pRR124, was synthesized using the cDNA synthesis kit and plasmid pRR525, which hybridized strongly to ³²P-labeled S10 RNA probe (Fig. 2), was selected.

cDNA of pRR525 was sequenced in both directions. As shown in Fig. 3B, the cDNA covered the 3' terminus of S10 (Fig. 3-A), but it was only about 150 bp in length and too short to connect with the sequence of pRR124.

For connecting sequences of pRR 124 and pRR 525, pRR 398, having about 900 bp in length, was selected from the cDNA library of RRSV genomic segments¹⁵⁾. Plasmid pRR398 was sequenced in both directions using primers which designed based on the sequence results from plasmids pRR124 and pRR525, respectively, and about 150 bp of nucleotides were determined. This sequence linked with those of pRR124 and pRR525.

Synthesis of full-length cDNA and its sequencing

In order to obtain a full-length cDNA of S10, the genome dsRNA was reverse transcribed from 3' terminus and then amplified by a PCR method. The cDNA clones were sequenced again to insure the accuracy of the previously determined sequence from the other cDNA clones described above. As shown in Fig. 1-C (5' terminus) and Fig. 3-C (3' terminus), terminal nucleotide sequences obtained from full-length cDNA were completely identical with those from the direct RNA sequencing and pRR525 cDNA.

Complete nucleotide sequence and predicted amino acid sequence

Figure 4 shows the complete nucleotide and predicted amino acid sequences of S10.

S10 is 1162 bp in length, and contains two predicted non-overlapping open reading frames (ORFs). The first ORF begins at 5' proximal AUG codon at nucleotide positions 20-22, and extends 12 codons to the UAG termination codon at positions 55-57. The second ORF extended 297 codons from the second AUG codon at positions 142-144 to the UAG stop codon at positions 1033-1035. It encodes a polypeptide with a predicted M_r of 32.4 K.

The function of this polypeptide is not clear. According to the published results^{3,5)}, the smallest structure protein of RRSV is 35 or 37 K. And it has been proven to be encoded by S9²²⁾. Therefore the polypeptide of S10 could be a non-structural protein, because no other structural proteins of comparable M_r has been reported. Homology search in NBRF-PIR (R37.0) failed to detect proteins of significant homology to the polypeptide.

It will be interesting to examine whether the upstream ORF is expressed *in vivo*. In the case of RDV, S1 has two ORFs, an upstream mini ORF and a major ORF encoding 8 and 1444 amino acids, respectively¹⁷⁾. Whether the mini ORF of RDV S1 is expressed *in vivo* has not been analyzed. But *in vitro* translation using the full-length cDNA showed the mini ORF affected the translational efficiency of the long ORF¹⁷⁾. Further

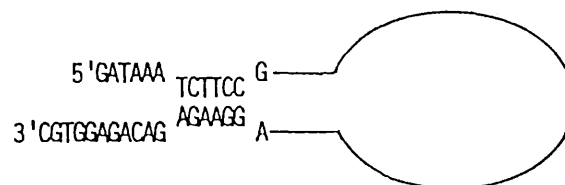


Fig. 5. Predicted terminal inverted repeat structure in RRSV S10.

studies are required to examine whether the upstream ORF of RRSV S10 is involved in the translational regulation of the long ORF.

Nucleotide sequence of S10 reveals a structure similar to the segment-specific inverted repeats reported near the termini of all segments of WTV¹⁾ (Fig. 5). The inverted repeat of S10 contains 6 bps adjacent to the conserved terminal sequences. In plant reoviruses, such putative segment-specific inverted repeat structures were also found in the terminal regions of all the segments sequenced so far^{9,21)}. These structures together with the conserved terminal sequences were considered to play a significant role in the sorting and assembly of segmented RNA genome in virus replication¹²⁾.

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和 文 摘 要

顔 瑾・須賀晴久・上田一郎・李 相龍・木村郁夫・四方英四郎：イネラギッドスタントウイルス (RRSV) ゲノムセグメント 10 のクローニングと全塩基配列

イネラギッドスタントウイルス (RRSV) のゲノムセグメント 10 (S10) のクローニングを行い、全塩基配列を決定した。末端領域の塩基配列はすでに報告した RNA の直接塩基配列決定法の分析結果と一致した。S10 は 1162 塩基対から成り、最も長い ORF は 891 塩基の翻訳領域を持ち、297 アミノ酸からなる 32.4 kDa のポリペプチドをコードする。長い ORF の上流に 12 アミノ酸をコードするミニ ORF が認められた。このミニ ORF の存在は逆転写-PCR 法で合成した S10 の全長 cDNA クローンの塩基配列分析でも確認された。また、5' と 3' 両末端に 6 塩基対の逆向き繰り返し配列も見いだされた。