J. Fac. Agr. Hokkaido Univ., Vol. 65, Pt. 4 : 351~357 (1992)

Rice dwarf virus genome segment 10 encodes a nonstructural protein : immunological evidence.

Takeshi MATSUMURA*, Ichiro UYEDA, Kazusato OHSHIMA, Jumanto HARJOSDARMO and Eishiro SHIKATA

Department of Botany, Faculty of Agriculture, Hokkaido University, Sapporo 060, Japan

*Hokkaido Green-Bio Institute, Kita 5, Higashi 15, Yubarigun Naganuma 069-13, Japan

(Received April 22. 1992)

Introduction

Rice dwarf virus (RDV) belongs to the genus *Phytoreovirus* and has 12 segmented double stranded RNAs as a genome. The complete nucleotide sequence of the genome segment 10 (S10) has been determined[3,11]. Analysis of the S10 sequence has demonstrated that it encodes a 39K protein. It was suggested that the S10 encoded a nonstructural protein, since the smallest structural protein of RDV was 43K[6]. We have also constructed a plasmid vector from which the authentic mRNA to the S10 could be transcribed *in vitro*[4].

In this paper, we describe the synthesis *in vitro* of a polypeptide expected from the nucleotide sequence analysis of the S10 and confirmation of its genome organization. An antiserum was produced against the bacterially expressed polypeptide. It was immunologically demonstrated that the S10 encodes a nonstructural polypeptide using the antiserum.

Materials and Methods

In vitro translation of RDV genome S10

Transcripts of RDV genome S10 were synthesized *in vitro* from plasmid pUMRD10 by T7 RNA polymerase[4]. This transcript was translated in a rabbit reticulocyte lysate (Amersham Co.) containing ^{35}S labelled methionine. Translation was done for 60 minutes at 30°C. Translation products were electrophoresed on 7.5% polyacrylamide-SDS gel according to the procedure described by Laemmli[1]. After electrophoresis, the gel was dried and autoradiographed.

Construction of Eschericha coli expression vector

A plasmid pUpRD28[5] which has a full-length cDNA of RDV genome S10 was digested with restriction enzyme *Hind* III at nucleotide 181 of the S10. Two units of DNA polymerase I klenow fragment and 1mM dNTPs were added to the

reaction mixture and it was incubated for 30 minutes at 37°C to make blunt ends at the restricted sites. The restriction fragment was further digested with *Sal* I at nucleotide 1144 of the S10. The cDNA was then inserted to the *Sma* I - *Sal* I site in the polylinker of an *E. coli* expression vector pRIT2T (Fig. 1). This plasmid pRIRD10 was able to express the S10 translation products as a fusion

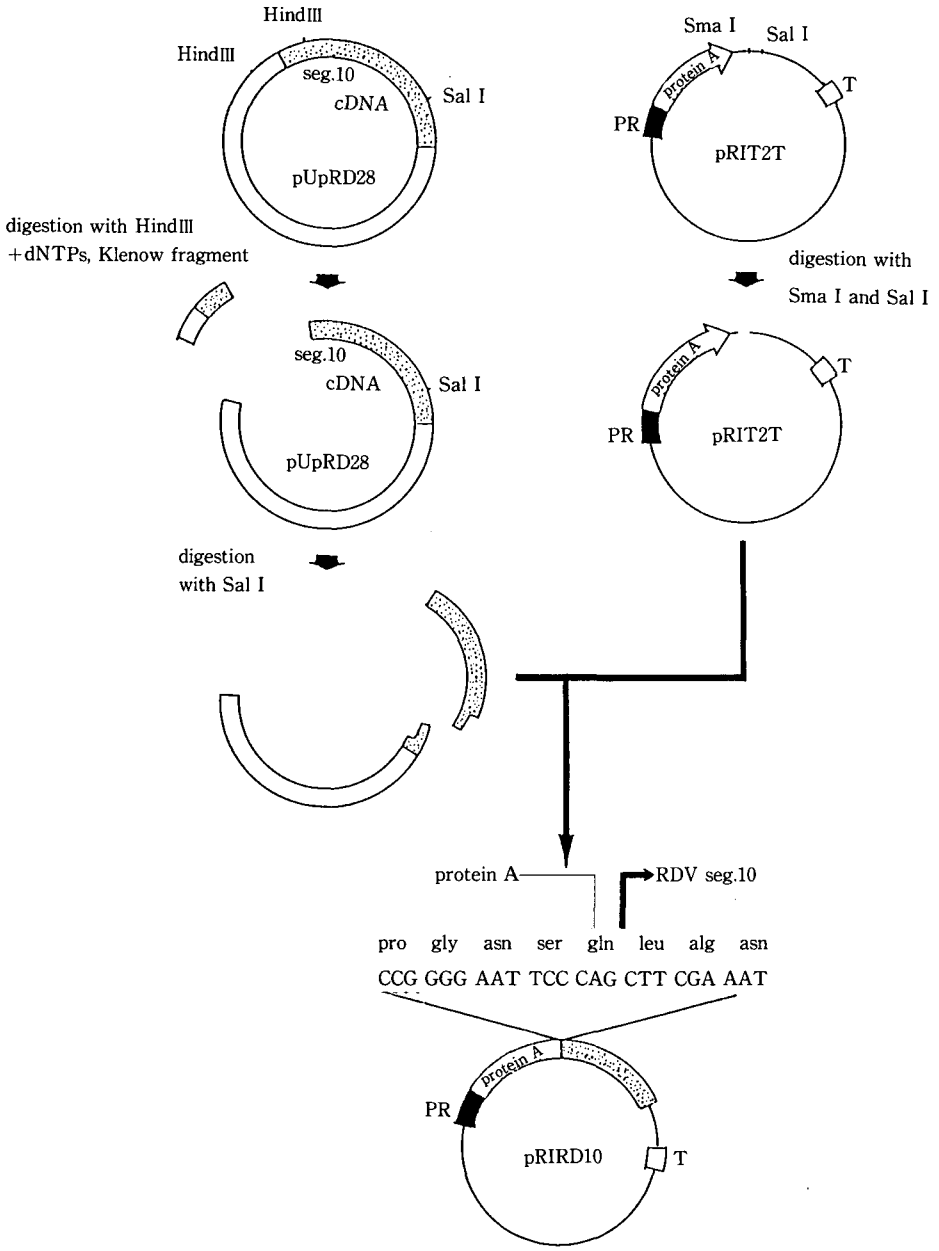

Fig. 1. Strategy for construction of pRD10.

protein with protein A under the control of PR promoter.

Expression and purification of fusion protein

E. coli strain N4830-1 was transformed with the plasmid pRIRD10 using the method by Maniatis *et al.*[2]. Expression of the fusion protein under control of PR promoter was performed as described by Nilsson *et al.*[7]. Proteins of the total cell lysate were analyzed by 10% polyacrylamide-SDS gel electrophoresis to confirm the expression of the fusion protein and the rest of the lysate was further purified by using IgG Sepharose 6 fast flow (Pharmacia Co.) according to a suppliers' recommendation.

Production of antiserum against fusion protein

Proteins bound to the IgG sepharose 6 fast flow were eluted by 0.5M acetate buffer (pH 3.4) and lyophilized and then were resuspended in the same buffer. The purified fusion protein was injected into a rabbit with a complete Freund adjubant. Two weeks after the second injection, the rabbit was bled and the serum was used in following experiments without further purification.

Western immunoblot analysis

Healthy and RDV infected rice leaves were homogenized in 0.1M phosphate buffer (pH 7.5) containing 0.01% triton X-100, and supernatants after centrifugation at 14,000 rpm for 20 minutes were loaded on 10% polyacrylamide-SDS gel. Proteins were electro-blotted to a sheet of nylon membrane by using Bio-Rad electro-blot system according to a manufacturer's protocol. The nylon membrane was saturated with the antiserum to the fusion protein in TST buffer (50 mM tris-HCl pH 7.6, 150 mM NaCl, 0.05% Tween 20) containing 5% skim milk. After washing with the TST buffer, the membrane was incubated in a solution of anti-rabbit goat peroxidase (8.75 μg/ml) conjugate in the TST buffer containing 5% skim milk at 37°C for 1 hour. After washing with TST buffer, proteins reacted with the antiserum were detected by an immunostain kit (Konica Co.).

Results

Analysis of translated products

Autoradiograph of the gel after electrophoresis showed a band specific to the translation programed by the transcripts from the plasmid pUMRD10 (Fig. 2). Molecular weight of this band was calculated to be about 39K and this was identical to the value of the longest open reading frame predicted from the nucleotide sequence. The result showed that first initiation codon of RDV genome S10 was actually used. And the fact that the smallest molecular weight of structural polypeptide of RDV was about 43K indicated that RDV genome S10 encoded a nonstructural polypeptide.

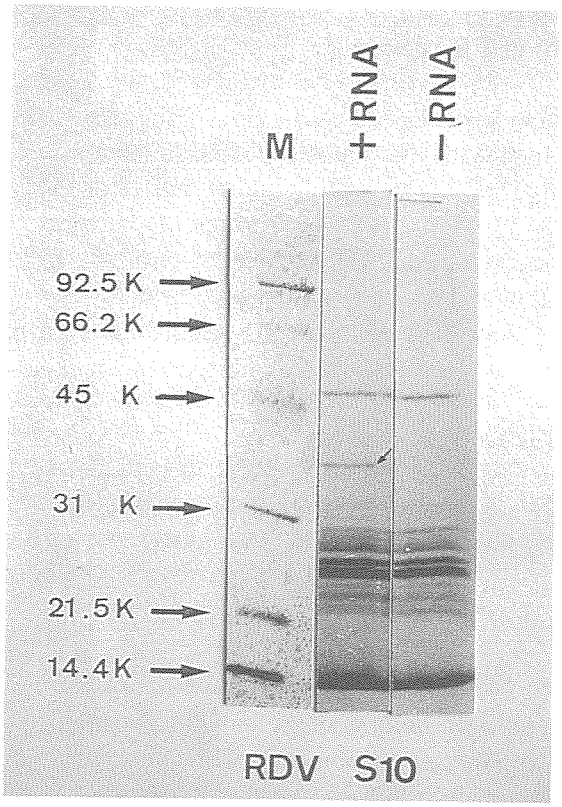

Fig. 2. Translation *in vitro* programed by RDV genome segment 10 transcripts synthesized from the plasmid pUMRD10. The products were analyzed on 7.5% polyacrylamide-SDS gel electrophoresis. The arrow on the middle lane indicates a band specific to the translation programed by RDV genome segment 10.

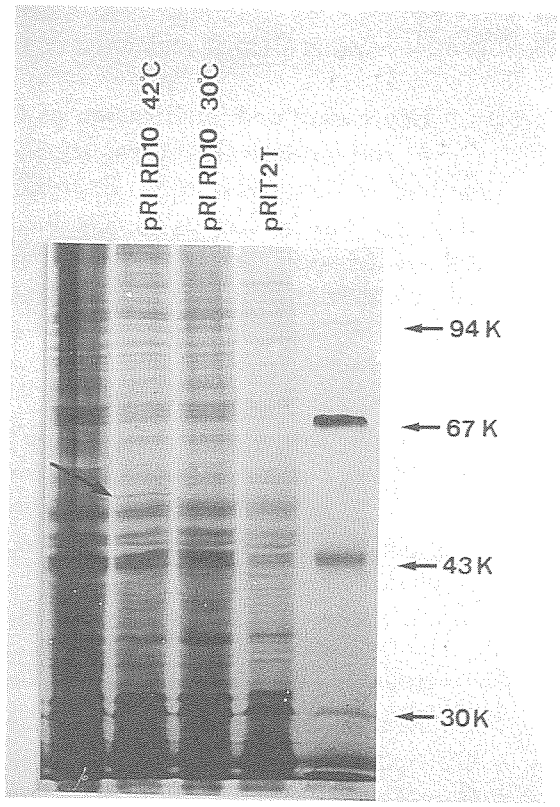

Fig. 3. Fusion protein of protein A-RDV genome segment 10. The product was analyzed on 10% polyacrylamide-SDS gel electrophoresis. Each lane received fusion proteins induced at 42 °C, not induced at 30 °C, and made from the plasmid DNA without insert (pRIT2T). The arrow on the 2nd lane from the left indicates a band of the fusion protein. The positions of protein size marker were indicated at right.

Detection of fusion protein expressed in E. coli

A band migrating at the position of 61K was specific to the extracts from the clone pRIRD10 when expression of the fusion protein was induced and analyzed by polyacrylamide-SDS gel (Fig. 3). The molecular weight of this band was the same as a putative translation product of the fusion protein, bacause of the molecular weight of the protein A in pRIRD10 was about 31K and the molecular weight of the inserted S10 polypeptide was calculated as about 30K.

Western blotting

Proteins purified by the IgG sepharose 6 fast flow column contained a fusion protein and some from *E. coli* (Fig. 4). The antiserum to the eluted proteins were predominantly reacted with the fusion protein in a western blotting analyses showing that the antiserum was able to detect the polypeptide encoded by the S10. This antiserum did not react with polypeptides from purified RDV particles (Fig. 5), showing that the S10 product is not a structural polypeptide. We also

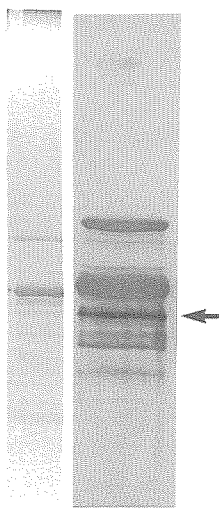

Fig. 4. Polyacrylamide-SDS gel (10%) and western blot of affinity-purified protein A-RDV segment 10 fusion proteins from *E. coli* containing plasmid pRIRD10. The left part was stained with silver stain (The GELCODE silver stain system, PIERCE chemicals), the right one was immuno-stained with antiserum against the fusion protein. Arrow indicates the position of the fusion protein.

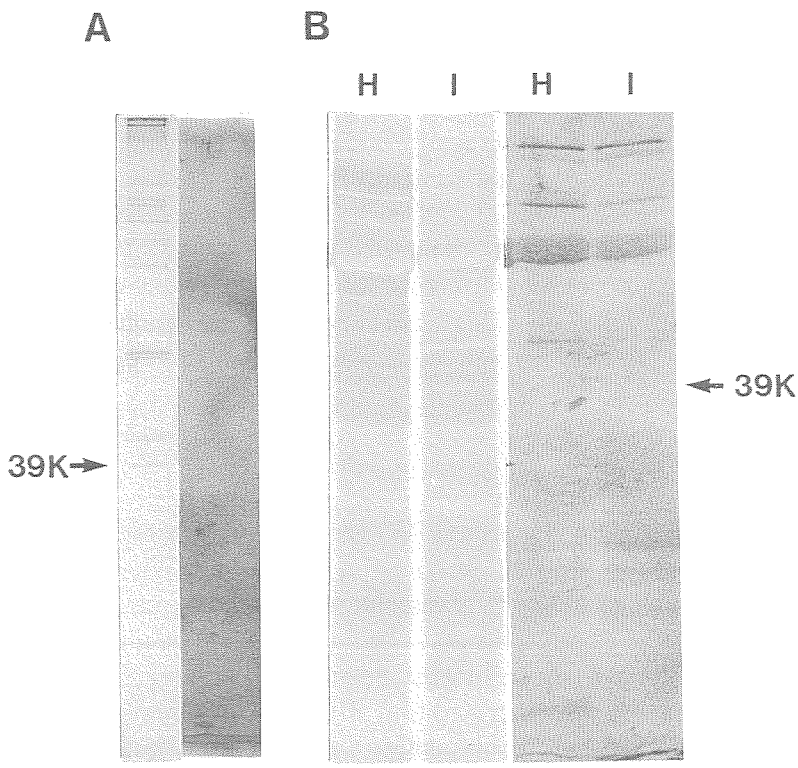

Fig. 5. Polyacrylamide-SDS gel (10%) and western blot analysis of RDV particles (A) and of rice leaf extracts from RDV infected and non-infected (B). The left part was stained with silver stain (The GELCODE silver stain system, PIERCE chemicals), the right one was immuno-stained with antiserum against fusion protein. Arrows indicate the position of MW. 39K.

attempted to detect the polypeptide in the infected rice plant. However, the antiserum failed to react with polypeptides from the infected plant. This is probably due to too low concentration of the polypeptide in the infected plant.

Discussion

The genome structure of RDV have already been investigated and coding assignment of some structural polypeptides to the genome segments were determined[8,9,10,11,12,13,14]. Up to 7 structural polypeptides have been reported for RDV[6]. Assuming that each genome segment encodes a single polypeptide, at least 5 polypeptides are non-structural viral proteins. These non-structural polypeptides have been detected neither *in vitro* nor *in vivo*.

In order to characterize the polypeptide encoded by S10, we synthesized S10 transcript completely identical to authentic one from the cDNA and it was used to program the *in vitro* translation in the rabbit reticulocyte lysate.

In this experiment, we confirmed that the longest open reading frame of S10, which was predicted from the nucleotide sequence, was translated *in vitro* and that it encoded a non-structural polypeptide.

If the translation product is non-structural protein, it is impossible to purify this protein from RDV particles and the antiserum to RDV particle may not react with this polypeptide. We therefore produced an antiserum to the bacterially expressed S10 polypeptide. The fact that the antiserum did not react with the viral structural polypeptide provided a further evidence for non-structural polypeptide. The approach employed in this report should be applicable to other non-structural polypeptides of RDV, which have not been so far detected or characterized.

Summary

Genome organization of rice dwarf virus (RDV) genome segment 10 (S10) was investigated by expressing its encoded polypeptide *in vitro*. When rabbit reticulocyte was programed by a full length transcript made from the cDNA clone pUMRD10[4], 39K polypeptide was produced. The molecular weight of the polypeptide was identical to that of the longest open reading frame predicted from the nucleotide sequence. This suggested that S10 encodes a non-structural polypeptide since the smallest structural polypeptide was reported to be 43K. An open reading frame of S10 from the cDNA clone pUpRD28[5] was fused to truncated protein A gene under control of the PR promoter in an *Escherichia coli* expression vector pRIT2T. The S10 fusion protein was purified and used for production of an antiserum. The antiserum reacted to the fusion protein but not to polypeptides from RDV particles. It was immunologically demonstrated that RDV genome S10 encodes a non-structural polypeptide.

Literature Cited

1. LAEMMLI, U. K. : Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **277** : 680-685. 1970
2. MANIATIS, T., E. F. FRITSCH and J. SAMBLOOK : "Molecular cloning" ; A laboratory manual. pp 545. Cold spring harbor laboratory 1982
3. MATSUMURA, T., I. UYEDA, T. SANO and E. SHIKATA : Complete nucleotide sequence of rice dwarf virus segment 10 transcript. Memoirs of the Faculty of Agriculture Hokkaido University **16** : 205-211. 1988
4. MATSUMURA, T., I. UYEDA and E. SHIKATA : Production of complete transcripts of rice dwarf virus genome segment 10 in vitro. Memoirs of the Faculty of Agriculture Hokkaido University **17** : 107-112. 1990a
5. MATSUMURA, T., I. UYEDA and E. SHIKATA : Tailored removal of flanking homopolymer sequences from rice dwarf virus segment 10 cDNA clone. Memoirs of the Faculty of Agriculture Hokkaido University **17** : 113-118. 1990
6. NAKATA, M., K. FUKUNAGA and N. SUZUKI : Polypeptide components of rice dwarf virus. Ann. Phytopath. Soc. Japan **44** : 288-296. 1978
7. NILSSON, B., L. ABRAHMSEN and M. UHLEN : Immobilization and purification of enzymes with staphylococcal protein A gene fusion vectors. EMBO J. **4** : 1075-1080. 1985
8. OMURA, T., K. ISHIKAWA, H. HIRANO, M. UGAKI, Y. MINOBE, T. TSUCHIZAKI and H. KATO : The outer capsid protein of rice dwarf virus is encoded by genome segment S8. J. gen. Virol. **70** : 2759-2764. 1989
9. SUZUKI, N., Y. WATANABE, T. KUSANO and Y. KITAGAWA : Sequence analysis of rice dwarf phytoreovirus genome segment S4, S5, and S6 : Comparison with the equivalent wound tumor virus segments. Virology **179** : 446-454. 1990
10. SUZUKI, N., Y. WATANABE, T. KUSANO and Y. KITAGAWA : Sequence analysis of the phytoreovirus segment S3 transcript encoding for the major structural core protein of 114 kDa. Virology **179** : 455-459. 1990
11. UYEDA, I., T. MATSUMURA, T. SANO, K. OHSHIMA and E. SHIKATA : Nucleotide sequence of rice dwarf virus genome segment 10. Proc. Japan Acad. **63** : 227-230. 1987
12. UYEDA, I., H. KUDO, T. TAKAHASHI, T. SANO, K. OHSHIMA, T. MATSUMURA and E. SHIKATA : Nucleotide sequence of rice dwarf virus genome segment 9. J. gen. Virol. **70** : 1297 -1300. 1989
13. UYEDA, I., H. KUDO, N. YAMADA, T. MATSUMURA and E. SHIKATA : Nucleotide sequence of rice dwarf virus genome segment 4. J. gen. Virol. **71** : 2217-2222. 1990
14. YAMADA, N., I. UYEDA, H. KUDO and E. SHIKATA : Nucleotide sequence of rice dwarf virus genome segment 3. Nucleic Acids Res. **19** : 6419. 1990