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MOLECULAR CLONING OF THE GENOME OF RICE RAGGED STUNT VIRUS

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I. Introduction

Plant reoviruses replicate both in plant and insect hosts[3]. In this respect, it is useful for studying genomes of plant reoviruses at the molecular level to elucidate a number of biologically important phenomena related to insect transmission and the regulation of viral genes and development in the host plants[12]. Rice ragged stunt virus (RRSV), the only member of subgroup 3, possesses 10 dsRNA segments as genome ranging from *Mr* 0.5×10^6 to 2.7×10^6 [4,8,13]. We recently reported that RRSV particles had RNA polymerase[17], and it transcribed full-length copies of all 10 genome segments *in vitro*[9].

In plant reoviruses, it has been reported that full-length copies of segment 12 in wound tumor virus (WTV) and segment 10 in rice dwarf virus (RDV) dsRNA genome segments were cloned into bacterial plasmid pBR 322[1,18]. But the cDNA synthesis from dsRNA requires more time and additional procedures in comparison with that from single-strand RNA. After the first cDNA strand is made, cDNA synthesis from dsRNA requires alkaline degradation of RNAs, annealing of the first cDNA strand of opposite polarities, and repair of single-stranded protruding ends by DNA polymerase I to make double-stranded cDNA. On the other hand, cDNA synthesis from single-strand RNA needs only one step reaction in a single tube to produce double-stranded cDNA after the first cDNA synthesis. Instead of establishing cDNA library from genomic dsRNA, we now report efficient and rapid cDNA synthesis from single-stranded RNAs which are transcribed by RNA polymerase associated with RRSV particles, and its cloning into pBR 322.

II. Materials and Methods

A. Isolation of single-stranded transcript RNA

RRSV was purified by a method of KAWANO *et al*[8]. The synthesis of single-stranded RNA was carried out from purified virus in the mixture of 1.2 ml contained 0.1 M Tris-HCl, pH 8.5, 8 mM $MgCl_2$, 1 mM NTPs, 4 mM phosphoenol pyruvate, 2 units of pyruvate kinase, 0.1 mM S-adenosyl-L-methionine, 0.6 mg/ml bentonite, 0.2 mg/ml chymotrypsin and 1 mg/ml RRSV for 4 hr at 37°C[9]. The reaction mixture was treated with 200 μg/ml proteinase K in 25 mM Tris-HCl, pH 7.5, 100 mM NaCl, 7.5 mM EDTA, 2.4% SDS for 20 min at 37°C[1]. The solution was extracted with phenol/chloroform (1 : 1) and the RNAs were precipitated by addition of 2.5 vol. of ethanol. The RNAs were incubated in 2 M LiCl overnight at 4°C and single-stranded RNA was then isolated from the precipitates after centrifugation.

B. cDNA synthesis

Synthesis of cDNA was carried out essentially as described for bovine preproenkephalin mRNA by GUBLER and HOFFMAN[6], except for polyadenylation step. Polyadenylated RNA was synthesized in the reaction mixture of 80 μl containing 50 mM Trsi-HCl, pH 8.0, 250 mM NaCl, 1 mM MnCl, 10 mM $MgCl_2$, 0.5 mM DTT, 2 mM ATP, 8 units of poly (A) polymerase (Takara Biomedicals) and 20 μg of the transcript RNA for 30 min at 37°C. The mixture was extracted with phenol/chloroform (1 : 1) and the products were precipitated with 2.5 vol. of ethanol. Polyadenylated RNA were ressolved in 0.1 ml of 50 mM Tris-HCl, pH 7.9, 10 mM $MgCl_2$, 30 mM KCl, 1 mM DTT, 5 mM dNTPs, 20 μCi of α-^{32}P dCTP, 10 μg of oligo $(dT)_{12\sim18}$, 1 μg of actinomycin D, 200 units of reverse transcriptase (Bethesda Research Laboratories), and incubated for 1 hr at 37°C. First strand cDNA was extracted with phenol/chloroform and precipitated with ethanol. For second-strand synthesis, first-strand cDNA was resuspended in 0.2 ml of 20 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 100 mM KCl, 5 mM β-NAD, 10 μg of BSA, 40 μM dNTPs, 1.7 units of RNase H, 40 units of DNA polymerase I (Takara Biomedicals) and 2.5 units of *Escherichia coli* DNA ligase. The mixture was incubated sequentially at 12°C for 1 hr and at 22°C for 1 hr. The products were extracted by phenol/chloroform and precipitated by ethanol.

C. Cloning into plasmid pBR 322

For annealing into pBR 322, cDNA was tailed with oligo (dC) in the reaction mixture of 100 mM Tris-HCl, pH 6.9, 50 mM sodium cacodylate, 2.5 mM

$CoCl_2$, 50 μM dCTP, 100 μM DTT, 5 units of terminal deoxynucleotide transferase (Takara Biomedicals) at 30°C for 15–25 min. After precipitation with ethanol, oligo (dC)-tailed cDNA was added together with 10 vol. of *Pst I*-cut and oligo (dG)-tailed pBR 322 (Bethesda Research Laboratories) in the mixture of 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.2 mM EDTA. After 3 min at 65°C, the mixture was held at 42°C for 2 h, cooled to 15°C overnight and then used to transform *E. coli* HB 101 by standard $CaCl_2$ procedure[14]. The resulting transformants were screened for tetracycline resistance and amplicillin sensitivity.

D. Hybridization

Two to five microgram of genome dsRNA separated by 5% polyacrylamide gel electrophoresis (PAGE) was hydrolyzed in 50 μl of 100% formamide at 100°C for 10 min. The RNA was percipitated by addition of 2 vol. of ethanol. Hydrolyzed RNA was then labeled with ^{32}P at 5′ termini in 50 μl mixture of 50 mM Tris-HCl, pH 7.5, 5 mM DTT, 0.01 mM spermidine, 0.1 mM $MgCl_2$, 0.01 mM EDTA, 40 μCi γ-^{32}P ATP and 10 units of T_4 polynucleotide kinase (Takara Biomedicals) at 37°C for 1 hr[11]. To detect virus-specific sequence in cloned DNA, dot blot hybridization was performed with ^{32}P-labeled genome dsRNA segments[7]. Recombinant DNAs were denatured at 100°C for 10 min and dotted onto nitrocellulose filter with dot blot apparatus (Bio-Rad). The filter was baked at 80°C for 2 hr, and pre-hybridized in 50% formamide, 5X SSC, 5X Denhardt's solution, 0.05 M sodium phosphate, pH 6.8, 0.1% SDS and 0.1 mg/ml yeast tRNA at 43°C for 7 hr. Hybridization was carried out at 42°C for 20 hr under a condition of 50 μl of pre-hybridization solution per 1 cm^2 and the solution possessed 10^5–10^6 cpm of probe per 1 ml. For southern-transfer hybridization, recombinant DNAs were digested with *Pst I* and electrophoresed in 1% agarose gel. The DNAs were denatured in 0.5 M NaOH/1.5 M NaCl, neutralized with equal volume of 1 M Tris-HCl, pH 8.0/3 M NaCl, transferred onto nitrocellulose filter and backed at 80°C for 2 hr[15]. Hybridization was carried out under the same condition as dot blot hybridization.

III. Results and Discussion

A. cDNA synthesis and molecular cloning

cDNA was synthesized from single-stranded RNAs which were transcribed by RNA polymerase associated with RRSV. The enzyme activity was stimulated by addition of chymotrypsin (0.2 mg/ml), and 0.7 mg of RRSV catalyzed the synthesis of 0.1 mg of single-stranded transcript RNA.

The transcript RNA was tailed at 3′ termini with oligo(A), and first strand cDNAs were synthesized. After synthesis of second strand cDNAs, the products were fractionated into 0.15 ml through a column of Bio-gel A-15 m (0.6×14 cm) equibilated in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1% SDS. When the first fraction contained cDNAs were detected by radioactivity, the following 4 fractions were collected and prepared for annealing to pBR 322. This procedure could remove small size class of cDNAs which are below about 400 base pairs.

As a result, approximately 1,000 transformants were generated on LB-agar containing tetracycline (12.5 μg/ml). Five hundred colonies were tested for ampicillin, and 493 colonies were ampicillin sensitive. The size of insert DNAs was estimated by 1% agarose gel electrophoresis of plasmids prepared by alkaline lysis method[2]. About 500-3,400 base pairs of insert DNAs were obtained and more than 90% of the insert DNAs ranged from 500 to 1,800 base pairs as shown in Fig. 1.

We have also attempted to make cDNAs from genome dsRNA of RRSV according to CASHDOLLAR *et al*[5]. This method yielded cDNAs at low

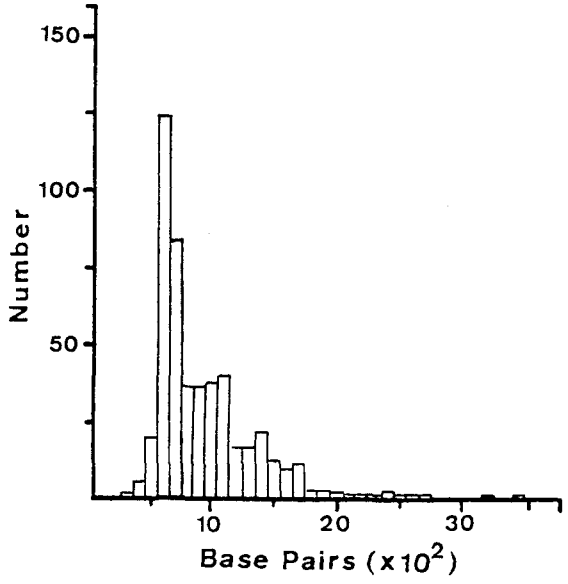

Fig. 1. Diagram of the size of insert DNAs. Approximately 1,000 transformants were generated from 20 μg single-stranded transcript RNA. Five hundred colonies selected randomly were tested for ampicillin and 493 colonies were screened. Base pairs of insert DNAs, using pRD of 600, 1,100 and 1,400 base pairs, were estimated by 1% agarose gel.

efficiency. Only about 10 transformants from 5 μg of dsRNA were obtained and the size of insert DNAs was 300–800 base pairs (data not shown). And in several other attempts to make cDNAs from total genomic RNAs, only cDNAs of particular part of genome segment 9 were frequently obtained. Part of the reason is probably a difficulty in denaturing and reannealing of genomic dsRNA in enzyme reactions.

B. Dot-blot hybridization with ^{32}P-labeled genome dsRNA segments

Genome dsRNA segments were separated individually by 5% PAGE in LEONING's buffer[10], and labeled with γ-^{32}P ATP. Ninety five out of 493 transformants were hybridized with each of ^{32}P-labeled genome dsRNA segment 7, 8, 9 and 10. Numbers of plasmid insert DNAs corresponding to genome dsRNA segments 7, 8, 9 and 10 were 10, 5, 3 and 4 respectively as shown in Fig. 2.

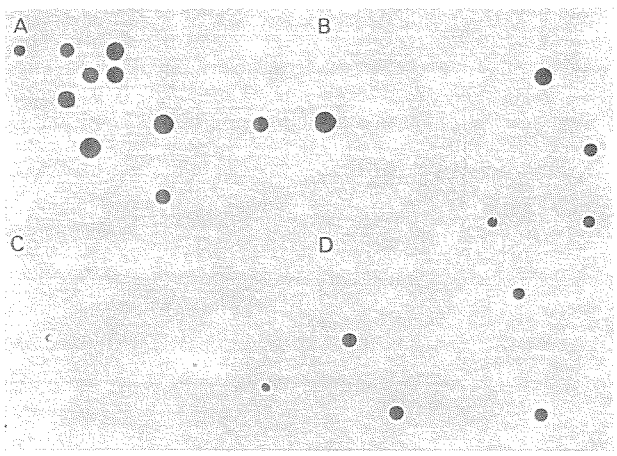

Fig. 2. Dot blot hy bridization with ^{32}P-labeled genome dsRNA segments. Ninety five plasmid DNAs were prepared randomly from 493 transformants by alkaline lysis procedure. The plasmid DNAs were dotted onto nitrocellulose filters (11×8 cm) with dot blot apparatus (Bio-Rad), and then hybridized individually with ^{32}P-labeled genome dsRNA segment 7 (A), 8 (B), 9 (C) and 10 (D).

C. Identification of cloned segment 9 gene

Recombinant DNAs from three clones of segment 9 gene selected by dot blot hybridization, designated as pRRS 9-26, 41 and 60, were digested with *Pst* I and electrophoresed in 1% agarose gel in 100 mM Tris base/100 mM boric acid/2 mM EDTA buffer. Each of the insert DNAs were estimated to 610, 620 and 900 base pairs in length (Fig. 3. A). The DNAs were

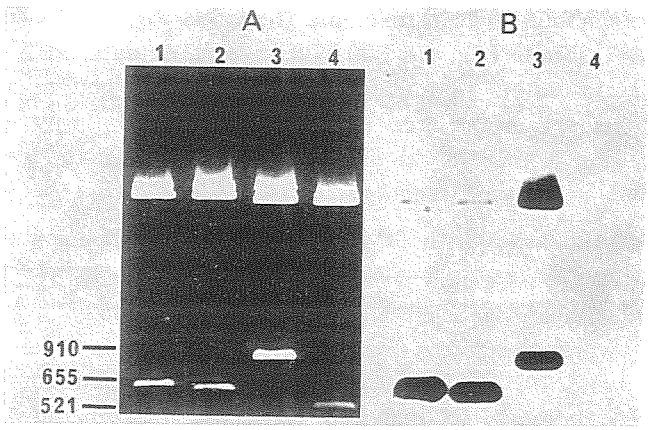

Fig. 3. Identification of insert DNAs corresponding to genome dsRNA segment 9. (A) Insert DNAs, pRRS9-26 (lane 1), pRRS9-41 (lane 2) and pRRS9-60 (lane 3), were digested with *Pst* I and electrophoresed in 1% agarose gel together with pRRS10 (lane 4). The figure on the left represent length in numbers of base pairs. Size markers were *Alu I* fragments of pBR 322. (B) Digested DNAs were transferred onto nitrocellulose filter (4×7 cm), and hybridized with ^{32}P-labeled genome dsRNA segment 9 as described in the text. Lane 1-3: pRRS 9-26, 41, 60 respectively; lane 4: pRRS 10 as control.

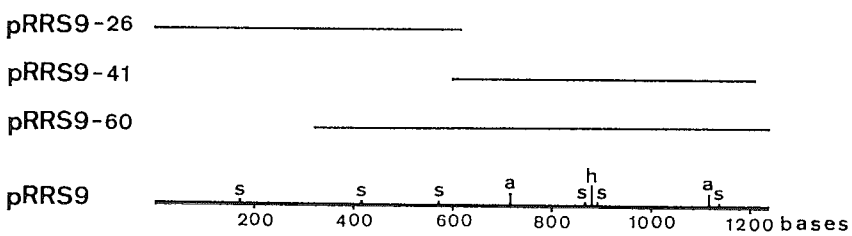

Fig. 4. Analysis of cleavage sites in cloned segment 9 gene. The insert DNAs were digested by restriction enzymes, and analysed the cleavage sites by 10% PAGE or 1.5% agarose-gel electrophoresis. pRRS 9: constructed frame of cloned segment 9 gene. a, h and s respresent the position of *Ava* I, *Hpa* II and *Sau*3*A* I cleavage site, respectively.

transferred onto nitrocellulose filter (4×7 cm), and prepared for hybridization as described in materials and methods. As expected, genome dsRNA segment 9 hybridized with pRRS 9, but not pRRS 10 (Fig. 3, B).

To determine the restriction enzyme cleavage sites, the DNAs were digested with *Ava*I, *Hpa*II and *Sau*3AI. In pRRS 9-26, only Sau3AI site is

found (Fig. 4.). The results show that pRRS 9-41 is overlapped with pRRS 9-60 and pRRS 9-26 is covered about 300 base pairs which are missed in pRRS 9-60. From these results, these recombinant DNAs cover apprroximately 1,240 base pairs. Base pairs of genome dsRNA segment 9 is estimated about 720 by molecular weight previously published[4,8].

We reported on sizing of genome dsRNA segments of RRSV by electron microscopy, and the molecular weight of segments 9 or 10 were higher than those previously reported[16]. The base pairs of genome dsRNA segment 9 was estimated to be about 1,200 by electron microscopy. Since cloned cDNAs cover 1,240 base pairs, it seems to be full-length of genome dsRNA segment 9.

IV. Summary

Single-stranded RNA, which was transcribed by RNA polymerase associated with rice ragged stunt virus (RRSV), was converted to cDNA and cloned into plasmid pBR 322. Approximately 1,000 transformants were generated from 20 μg of transcript RNAs and about 90% of insert DNAs had 500-1,800 base pairs. Virus specific DNAs were screened by dot blot and southern transfer hybridization. Three of 96 recombinant DNAs were corresponded to genome dsRNA segment 9. Each of the size was 610, 620, and 900 base pairs. Analysis of the cleavage sites by restriction enzymes showed that the DNAs covered 1,240 base pairs in length. Thus it seemed to be full-length copy of genome dsRNA segment 9.

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