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Sequence Analysis and GTP-Binding Ability of the Minor Core Protein P5 of *Rice Gall Dwarf Virus*

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Abstract

Analysis of the nucleotide sequence of the genome segment S5 of *Rice gall dwarf virus* (RGDV), which belongs to the genus *Phytoreovirus*, revealed that this segment encodes a putative protein of approximately 91 kDa. Antiserum raised against the protein reacted with a minor structural protein of similar size in RGDV, demonstrating that S5 encodes the structural protein of RGDV. The protein named P5 appeared to be a component of viral core particles. Although no overall homology was found to any other proteins, including those of animal-infecting reoviruses, P5 of RGDV exhibited a significant homology to P5 of *Rice dwarf virus* (50%), which might be a guanylyltransferase, and to P5 of *Wound tumor virus* (55%). These results, together with the observation that P5 bound guanosine triphosphate suggest that P5 of RGDV shows a guanylyltransferase activity and, moreover, that plant-infecting reoviruses have a similar functional and/or structural organization to that of animal-infecting reoviruses.

Discipline: Plant disease
Additional key words: RGDV, *Phytoreovirus*

Introduction

One of the notable properties of the viruses in the family *Reoviridae* is the presence in their genomes of genes for RNA-dependent RNA polymerase. The genus *Phytoreovirus*[2] including *Wound tumor virus* (WTV), *Rice dwarf virus* (RDV) and *Rice gall dwarf virus* (RGDV) is also characterized by this activity[9, 25, 31]. The mRNA transcribed by the encoded polymerase has a cap structure at its 5' end[27]. Therefore, it is likely that a guanylyltransferase that can catalyze the formation of a $m^7G'ppp^{5'}$Am-cap at the 5' terminus of transcripts is present in the *Phytoreovirus* particles, as in the case of animal reoviruses[5, 18, 26]. In RDV, the P5 protein encoded by the S5 segment of the dsRNA genome binds GTP covalently, suggesting that P5 of RDV can be a guanylyltransferase[29]. Thus, it appears that P5 has a specific function, even though its relative ratio in viral particles is very low (unpublished data, Omura) and was not detectable in our earlier analysis of the structural proteins of RGDV[20].

In RGDV and RDV which are similar in morphology, the molecular sizes and the molar ratios of structural proteins are also similar. However, RGDV and RDV differ in terms of their biochemical, structural and molecular biological properties. The optimum temperature for the activity of the RNA-dependent RNA polymerase is 25ºC

The nucleotide sequence reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases under the accession number D76429.

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in RGDV and 35ºC in RDV[31]. The P2 protein encoded by the genome segment S2 is essential for viral infection of insect vector cells and can be removed by organic solvents from RDV but not from RGDV[17, 23]. RGDV propagation is restricted to phloem-related cells in infected plants, while RDV is systemic in infected rice plants[19]. Comparative analysis at the molecular level of these closely related viruses would enhance our understanding of the relationships between the structure of component protein and virus properties, which ultimately may lead to disease control. To confirm the presence of a protein which was not detectable in our earlier study and to gain more insight into the structure-function relationships in the genus *Phytoreovirus*, we analyzed the S5 genome segment of RGDV and examined the properties of the encoded protein.

Determination of nucleotide sequence

A cDNA library of the segments of RGDV genome that had been cloned into pBR322[10] was used for the selection of transformants that contained S5 cDNA by colony hybridization with a ^{32}P-labeled S5 dsRNA probe. Among the plasmids that contained S5 cDNA, we selected 2 long cDNAs and recloned them into pBluescript II KS(+) (Stratagene, La Jolla, CA, USA) in both orientations. Detection of the cDNA in the plasmids at intervals of about 200 base pairs was induced by digestion with exonuclease III (Takara Shuzo, Tokyo, Japan). The nucleotide sequence of each insert was determined using a Dye Primer Cycle Sequencing Kit (Perkin-Elmer Applied Biosystems, Foster City, CA, USA) and automatic sequencers (models 373A and 377; Perkin-Elmer Applied Biosystems). All the regions of the cDNAs were sequenced at least twice in each direction.

Nucleotide sequences were assembled and analyzed with DNASIS (Hitachi Software Engineering Co., Tokyo, Japan) and GENETYX (Software Development Co., Tokyo, Japan) software packages, and a search for proteins homologous to the predicted protein encoded by RGDV S5 was performed with the BLAST program. Two cDNAs analyzed were identical.

The S5 segment of RGDV was 2,542 bp long and its G + C content was 39.8%. The terminal sequences were 5'-GGUAAUUU---AAAAAUAGU-3'. These sequences were highly homologous to the previously reported terminal sequence of other segments of RGDV[10, 11, 17, 21]. An imperfect inverted repeat of 9 nucleotides (nt) was detected adjacent to each conserved terminal sequence between positions 3 and 12 and positions 2,540 and 2,530. The sequence of the cDNA contained one long open reading frame (ORF) that started at position 13 and extended for 2,412 nt; the ORF was followed by a 3' noncoding region of 130 nt. This long ORF encoded a putative polypeptide of 799 amino acids with a calculated molecular mass of 90,870 Da (ca. 91 kDa). No other ORFs, including the ORFs on the negative strand, encoded a polypeptide of more than 61 amino acids.

Homology searches

The 91-kDa protein of RGDV was 50 and 55% homologous at amino acid levels to the 90-kDa protein of RDV[8, 28], which appears to be a guanylyltransferase[29], and to the 90-kDa protein of WTV[1], respectively. Long regions of identical respective amino acid sequences were found in the corresponding regions of the 3 proteins. When conservative changes of the type reported by Dayhoff et al[4]. were taken into account, the homology between the 91-kDa protein of RGDV and 90-kDa proteins of RDV and WTV increased to 67 and 71%, respectively. Other proteins, including those of viruses in the family *Reoviridae*, whose sequences had been deposited in the database did not exhibit any significant homology to the 91-kDa protein of RGDV. These results suggested that the 91-kDa polypeptide deduced from the nucleotide sequence of S5 might be the counterpart of the P5 proteins of RDV and WTV. There was no apparent serological cross-reaction in the Western-blotting analysis of the 91-kDa proteins of RGDV and the P5 of RDV using the antiserum to P5 of RGDV prepared in this study and described below (data not shown) in spite of their strongly conserved primary structures.

Baculovirus expression

To verify the coding assignments for S5 of RGDV, we expressed the deduced 91-kDa protein in Sf-9 cells and compared its electrophoretic mobility with those of structural proteins of RGDV. We amplified the cDNA that corresponded to the longest ORF, namely, nt 13 to nt 2,412 of the S5 genome segment of RGDV by the polymerase chain reaction (PCR) using a 5' oligonucleotide primer (5'-GGGGTAGATCTGCTATGCAGGCGAAAACCA-3') that contained a *Bgl*II site (underlined) and a 3' oligonucleotide primer (5'-ACGCAACCGTAGGAAAGCTTCTGTCGTTTG-3') that contained a *Hin*dIII site (underlined). Primers were based on the newly established sequence of the cDNA. The product of PCR was digested with *Bgl*II and *Hin*dIII and isolated by agarose gel electrophoresis. The fragment was cloned into the pBlueBacIII expression vector (Invitrogen, San Diego, CA, USA) that had been digested with *Bgl*II and *Hin*dIII. The integrity of the inserted sequence was con-

firmed by the digestion with restriction enzymes. The plasmid and linearized AcMNPV were co-transfected to Sf-9 cultured cells according to the instructions from Invitrogen. A recombinant baculovirus with cytopathic effects that were not associated with the formation of polyhedral inclusion bodies was propagated and used to express the 91-kDa protein in Sf-9 or High Five cells (Invitrogen, San Diego, CA, USA), as described in the protocol from the manufacturer. Infected cells were collected 6 days after the infection and pelleted by low-speed centrifugation. The pelleted cells were mixed with a protein-dissociation buffer[12] and the proteins were resolved by SDS-7.5% polyacrylamide gel electrophoresis (PAGE) and stained with Coomassie Brilliant Blue (CBB). A major band with the mobility of a protein of approximately 75 kDa was detected on the SDS-PAGE gel (data not shown). The product of S5 of RGDV expressed by the recombinant baculovirus migrated more rapidly than expected from the size of the deduced protein. The reason for this discrepancy remains to be determined.

Determination of amino acid sequence

To confirm that the expressed protein with the unexpected mobility was derived from S5 of RGDV, we analyzed the amino acid sequence of the protein. After SDS-7.5% PAGE, the band of the protein was cut from the gel and electroeluted. The purified protein was digested with *Staphylococcus aureus* V8 protease (Wako, Osaka, Japan; 0.1 g/L). Peptide fragments were resolved by SDS-18% PAGE, transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon-P; Millipore, Yamagata, Japan) and subjected to amino acid sequence analysis on an automated protein sequencer (model 492; Perkin-Elmer Applied Biosystems, Foster City, CA, USA). The amino acid sequence of one polypeptide fragment was FFGNQPYSII, corresponding to residues 349–358 of the polypeptide deduced from S5 and confirming that the expressed protein had originated from S5 of RGDV.

Production of antiserum and detection by Western-blotting

To confirm that S5 encodes a structural protein of RGDV, we raised antiserum against the expressed protein and allowed it to react with viral structural proteins. The band of the expressed protein on the gel after SDS-7.5% PAGE was excised and the protein was electroeluted in 10-fold diluted Tris-glycine-SDS buffer[12] in a protein-extraction unit (Nihon-Eido, Tokyo, Japan). Antiserum was raised in a rabbit, which was immunized 4 times with approximately 4 mg of total protein, as described previously[20]. Complete Freund's adjuvant was included in the second immunization, which was given by intramuscular injection. Serum was collected 7 days after the last injection. Western blotting analysis was performed after transferring viral proteins from the gel to a PVDF membrane using an electrotransfer unit (Nihon-Eido, Tokyo, Japan). The membrane was placed in a blocking buffer, which consisted of 3% bovine serum albumin in TBS (0.02 mol/L Tris, 0.5 mol/L NaCl, pH 7.5), for 1 h. Then the membrane was incubated for 16 h with 4,000-fold diluted antiserum in TBS that contained 0.05% Tween-20 (TBS-T). After the membrane had been washed in TBS-T, the blocking buffer was applied that contained 1,000-fold diluted alkaline phosphatase-conjugated antibodies raised in goat against rabbit immunoglobulin G (Jackson Immuno Research Town, PA, USA). The membrane was developed with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate as substrate. As shown in Fig. 1, the antiserum reacted specifically with a minor structural protein of RGDV. All our results together demonstrated that S5 of RGDV encodes a minor capsid protein of 91 kDa. We designated this protein as P5.

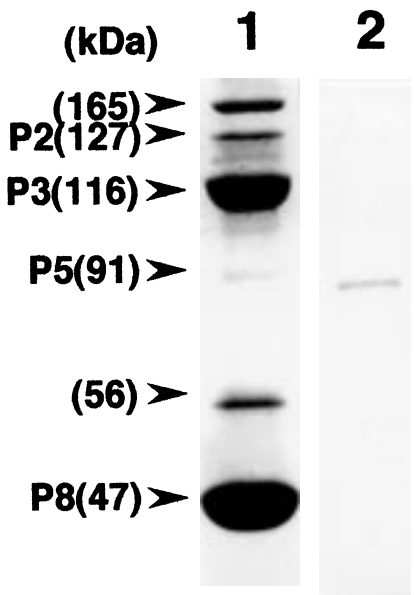

Fig. 1. Detection of P5 protein of RGDV by Western blotting

Lane 1: Proteins from purified RGDV particles, Lane 2: Western blotting analysis of purified RGDV particles using antiserum against the S5 product of RGDV that was expressed by a recombinant baculovirus. Positions of viral structural proteins are shown on the left.

Localization of P5 protein in particles

To determine the location of P5 in viral particles, we separated core particles from outer capsid proteins, as described elsewhere[30], and subjected both preparations to SDS-10% PAGE[12]. The RGDV[19] used for this analysis had been purified with CCl4 in the same way as reported previously for RDV[22]. Infected rice leaves were macerated in a meat chopper. Then, the slurry of chopped leaves was subjected to differential centrifugation and to consecutive sucrose density gradient centrifugations first on 10–40% and then on 40–60% sucrose. The final pellet after high-speed centrifugation of the purified virus was resuspended in a 0.1 mol/L solution of histidine that contained 0.01 mol/L MgCl2, pH 6.2, and stored at –70ºC.

Purified viral particles were incubated for 5 min in a 0.1 mol/L solution of histidine (pH 6.2) that contained 1.2 mol/L MgCl2 and centrifuged for 10 min at 200,000 × g in a TL-100 rotor (Beckman, Palo Alto, CA, USA). The supernatant and the pellet suspended in the solution were separately centrifuged again. Final pellets containing core particles were analyzed by SDS-10% PAGE with subsequent staining with CBB. As shown in Fig. 2, P5 was found in the preparation of core particles. Thus, P5 appeared to be a component of the core of RGDV.

GTP-binding ability of P5

The strong homology of P5 of RGDV to that of RDV, which binds nucleotide triphosphate[28], suggested that P5 of RGDV also had the ability to bind GTP. To examine this possibility, we incubated 20 µg of purified RGDV particles, in a total volume of 20 µL, in 0.02 mol/L histidine-HCl (pH 6.2), 1.0 mol/L DTT, 0.01 mol/L $MgCl_2$ and 0.17 µmol/L [α-^{32}P], GTP (3,000 Ci/mL; Amersham Pharmacia Biotech, Little Chalfont, UK) as described earlier[29]. Among 6 capsid proteins of RGDV, only P5 protein bound [α-^{32}P] GTP (Fig. 3). The radioactivity remained associated with the protein even after boiling in the presence of 2% SDS and 1% mercaptoethanol. The binding was detected only when viral particles that were not previously denatured were used. Dissociation of RGDV particles prior to the incubation with [α-^{32}P] -labeled GTP prevented the labeling of the polypeptide.

Conclusion

The results indicate that P5 proteins, the structural proteins of *Phytoreovirus* that are present at the lowest levels, display a GTP-binding ability and are likely to

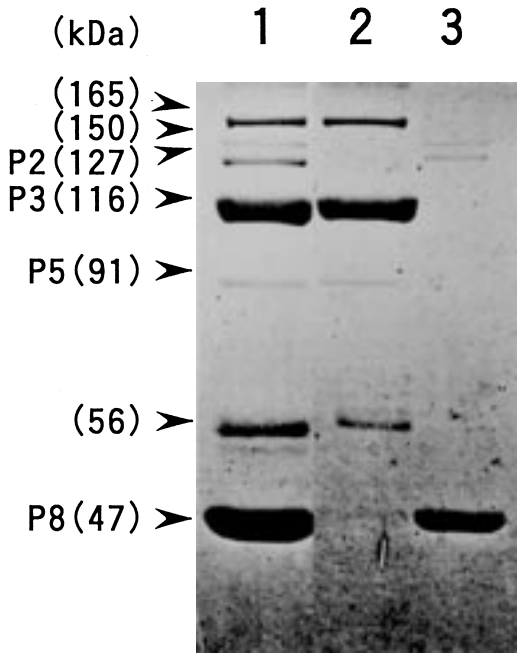

Fig. 2. Location of P5 in RGDV particles
Lane 1: Proteins from intact particles, Lane 2: Proteins from core particles, Lane 3: Proteins from a supernatant fraction after high-speed centrifugation of $MgCl_2$-treated RGDV particles (1.2 M $MgCl_2$). All the samples were fractionated by SDS-10% PAGE and the gel was stained with CBB.

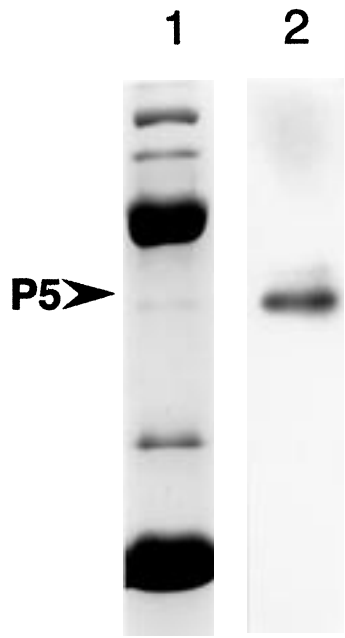

Fig. 3. Binding of GTP by P5 of RGDV
Lane 1: Proteins from intact particles after separation by SDS-7.5% PAGE, Lane 2: Proteins after the GTP-binding assay in which RGDV particles were incubated with radiolabeled GTP. The position of the P5 protein of RGDV is indicated by an arrow on the left.

consist of guanylyltransferase[29]. Among the proteins isolated to date from animal-infecting viruses in the family *Reoviridae*, reovirus λ2[3, 6, 13, 16], rotavirus VP3[5, 15], and bluetongue virus VP4[7, 14, 24, 26] binding GTP are likely to be guanylyltransferases, and all these are minor structural proteins as in the case of RGDV. Thus, the determination of the biochemical properties of the P5 of RGDV obtained in the present study should contribute to further analysis of the viral infection process in infected hosts cells.

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