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Rice Grassy Stunt Tenuivirus Nonstructural Protein p5 Interacts with Itself To Form Oligomeric Complexes In Vitro and In Vivo

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We investigated the interaction of *Rice grassy stunt tenuivirus* (RGSV) nonstructural protein p5, a protein of 22 kDa encoded on vRNA 5, with all 12 RGSV proteins by using a GAL4 transcription activator-based yeast two-hybrid system. The p5 protein interacted only with itself and not with any other viral protein; the interacting domains were localized within the N-terminal 96 amino acids of p5. The p5-p5 interaction was reproduced in an Sos recruitment-mediated yeast two-hybrid system as well in by far-Western blots. Native p5 protein extracted from RGSV-infected rice tissue was detected in a large complex with a molecular mass of approximately 260 kDa composed of 12 molecules of p5 or a p5 oligomer with an unidentified host factor(s).

The genome of *Rice grassy stunt tenuivirus* (RGSV), a member in the genus *Tenuivirus* (12) and an important rice pathogen in East, Southeast, and South Asian countries (8), consists of six ambisense RNA segments containing a total of 12 open reading frames (ORFs) (13, 16, 17). RGSV replicates and is transmitted by a brown planthopper (*Nilaparvata lugens* Stal) (9). So far, little has been known about the functions of the individual RGSV proteins, except for the 339-kDa RNA-dependent RNA polymerase (RdRp) encoded on the complementary strand of RNA 1 (cRNA 1) and the 36-kDa nucleocapsid protein (N) encoded on cRNA 5 (Fig. 1), both of which are found along with genomic and possibly complementary RNAs as thin filamentous ribonucleoprotein (RNP) particles. Recently, we showed that the 22-kDa p5 protein encoded on the virus genomic strand of RNA 5 (vRNA 5) accumulates in large amounts in both RGSV-infected rice leaves and viruliferous brown planthoppers, while the 23-kDa p2 protein encoded on vRNA 2 and the 21-kDa p6 protein encoded on vRNA 6 were preferentially expressed in infected rice leaf tissues rather than in viruliferous insects (5). We hypothesized that p5 has an essential role in virus infection in both the plant and insect hosts (5).

In this study, we investigated possible interactions between the p5 protein and the other RGSV proteins by using a GAL4 transcription activator-based yeast two-hybrid system (4, 6). We found that p5 interacts with itself through domains located in the N-terminal half of the protein, but does not interact with any other RGSV protein with this yeast two-hybrid system. The p5-p5 interaction was confirmed by an Sos recruitment-mediated yeast two-hybrid system (1, 2) as well as by far-Western blots, indicating that the strength of the p5-p5 interaction is significant and suggesting that the interaction is biologically significant as well.

p5-p5 interaction in the N-terminal region detected by a GAL4 transcription activator-based yeast two-hybrid system. Interactions between the p5 protein and all 12 RGSV proteins were examined by using a yeast two-hybrid system based on the GAL4 transcription activator (4, 6) (MatchMaker 2; Clontech). In this system, the yeast GAL4 transcription activator has been separated into two functional domains: (i) the DNA binding activity present on plasmid pAS2-1 with the *TRP1* gene as a selectable marker and (ii) the transcription activation property present on plasmid pACT2 with the *LEU3* gene for selection. The two genes of interest are expressed in pAS2-1 and pACT2 as fusion proteins. A direct interaction between the two foreign gene products in the yeast nucleus activates the GAL4 transcription activator and leads to the expression of the reporter genes *lacZ* and *HIS3*. This interaction can be monitored either by the ability of the transformants to grow on synthetic medium lacking Leu, Trp, and His or by direct assays of β-galactosidase activity. For these experiments, except for the 339-kDa RdRp gene and a 94-kDa protein gene on cRNA 2, each of the 10 ORFs was cloned as a full-length cDNA into both pACT2 and pAS2-1 plasmids so that each protein could be expressed as a fusion protein with either the GAL4 activation domain (AD) or the DNA binding domain (BD), respectively. The 339-kDa RdRp gene was divided into five overlapping segments, whereas the 94-kDa protein gene was divided into three overlapping segments, each of which was cloned into pACT2 and pAS2-1 (Fig. 1). Each cDNA insert was derived from the previously cloned cDNA that had been used for nucleotide sequence analysis (13) or from reverse transcription-PCR products from RNA extracted from RNP particles purified by sucrose density gradient centrifugation. For cloning of inserts into pACT2 and pAS2-1 plasmids, the PCR primers were designed to add a restriction endonuclease recognition sequence to the 5′ end of the insert so that it could be cloned in frame to AD or BD genes and to add a termination codon at the end of each ORF.

Recombinant plasmids were transformed into *Escherichia coli* strain MC1061. Plasmid DNA was isolated by a modified

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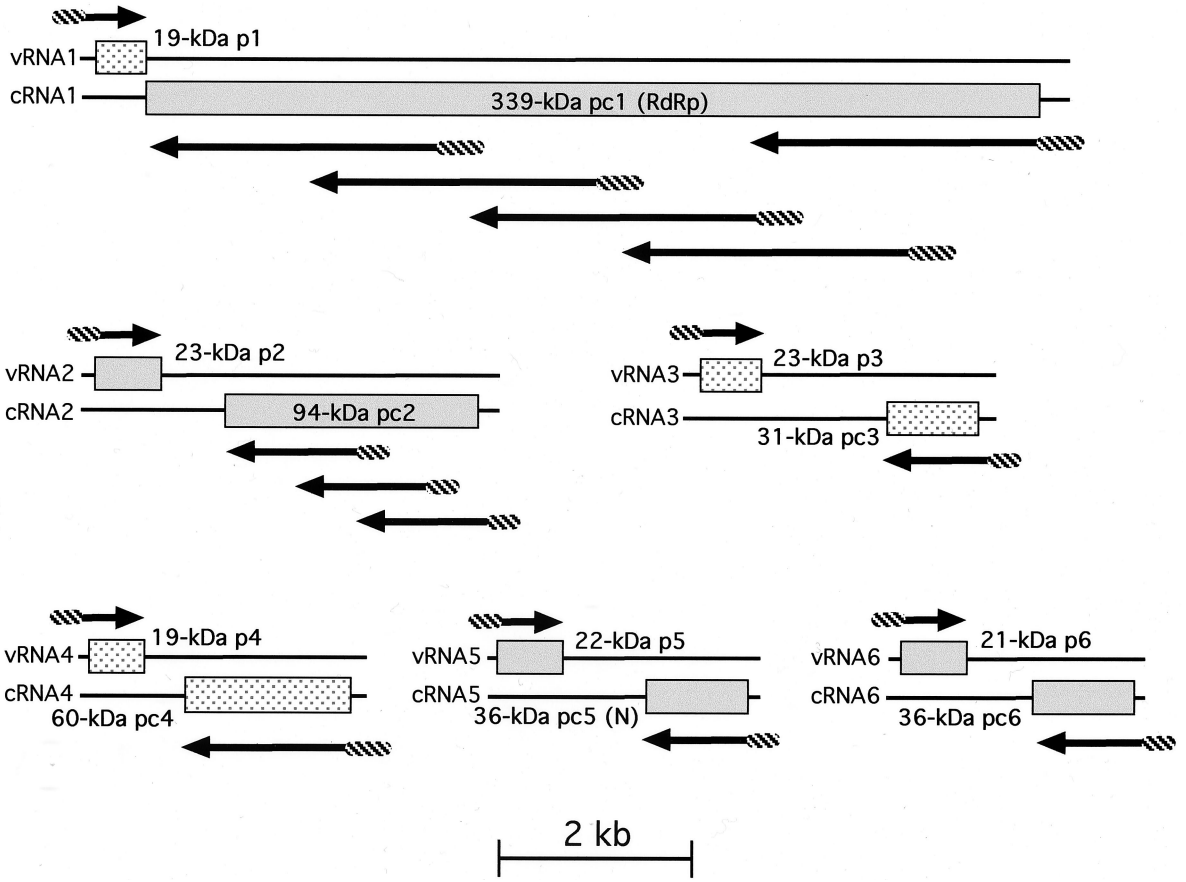

FIG. 1. Genome structure of RGSV and location and orientation of RGSV cDNA inserts cloned into pACT2 and pAS2-1 plasmids. vRNA, virus genomic strand; cRNA, complementary strand RNA; gray rectangular boxes, ORFs common to all tenuiviruses; dotted rectangular boxes, ORFs unique to RGSV. Arrows indicate the RGSV cDNA inserts cloned into the two plasmids from 5′ to 3′ toward the arrowhead and fused to the AD or BD indicated by shaded bars on the other end of the arrow.

boiling method (10). Competent cells of *Saccharomyces cerevisiae* strain Y190 were transformed simultaneously with pACT2 and pAS2-1 recombinant DNAs by the lithium acetate method (7). The synthetic media used for selection of yeast transformants consisted of 1.5% agar, yeast nitrogen base (Gibco BRL), 2% glucose, and essential amino acids and nucleotides, one or more of which were omitted, according to a protocol provided by Clontech. Y190 yeast cells cotransformed with pACT2 and pAS2-1 recombinant plasmids were plated onto the synthetic medium lacking Leu, Trp, and His and supplemented with 25 mM 3-aminotriazole (Sigma/Aldrich, St. Louis, Mo.). Plates were incubated at 30°C for up to 5 days. Interactions between two RGSV proteins were initially indicated by colony formation on the selective medium and confirmed by detection of β-galactosidase activity in a filter colony lift assay with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) as a substrate (3). Yeast cells cotransformed with pAS2-1 and pACT2 without inserts were used as negative controls, whereas those cotransformed with pTD1-1 (AD-simian virus 40 [SV40] large T antigen) and pVA3-1 (BD-mouse p53) were used as a positive control (data not shown).

Y190 yeast cells transformed only with recombinant pAS2-1 DNAs were tested for self-activation on synthetic medium lacking Trp and His. A 19-kDa p1 protein (encoded on vRNA 1) and the N-terminal 1,000-amino-acid region of the RdRp protein showed weak self-activation. The 31-kDa pc3 encoded on cRNA 3 showed strong self-activation, probably due to its highly acidic nature (21% acidic amino acids) (Table 1). On the other hand, Y190 yeast cells transformed with recombinant pACT2 DNAs did not grow on synthetic medium lacking Leu and His, indicating that no DNA-binding activity was detectable for any RGSV protein in this system (Table 1).

When Y190 yeast cells were transformed with pAS2-1/p5 which contains the p5 gene fused with the BD region and one of pACT2 derivatives expressing an RGSV protein fused with the AD, only the combination of pAS2-1/p5 and pACT2/p5 resulted in growth of cells on the synthetic medium lacking Leu, Trp, and His (Table 1). Similarly, when pACT2/p5 was transformed into Y190 cells with one of pAS2-1 derivatives expressing an RGSV protein fused with the BD, there was no significant cell growth observed on synthetic medium lacking Leu, Trp, and His, except for cotransformants with pACT2/p5 and pAS2-1/p5 (Table 1). The p5-p5 interaction was confirmed by β-galactosidase colony lift assay; colonies formed by cotransformants turned dark blue within 2 h (Fig. 2B) (1). These positive results in both directions strongly indicated that p5 domains in AD- and BD-fusion proteins interacted in the transformed Y190 yeast nucleus.

TABLE 1. Growth of Y190 yeast cells transformed with pAS2-1 and pACT2 plasmids expressing RGSV proteins on synthetic medium plates lacking Leu, Trp, or His

Fused protein in RNA segment[a]	Result for plasmid construct(s) (selection medium)[b]			
	pAS2-1/X (−Trp, −His)	pACT2/X (−Leu, −His)	pAS2-1/p5 and pACT2/X (−Leu, −Trp, −His)	pACT2/p5 and pAS2-1/X (−Leu, −Trp, −His)
None	−	−	−	−
RNA 1				
p1 (full length)	+	−	−	NT
pc1.N (1–1000)	+	−	−	NT
pc1.M1 (501–1500)	−	−	−	−
pc1.M2 (976–1950)	−	−	−	−
pc1.M3 (1476–2450)	−	−	−	−
pc1.C (1926–2925)	−	−	−	−
RNA 2				
p2 (full length)	−	−	−	−
pc2.N (1–411)	−	−	−	−
pc2.M (205–615)	−	−	−	−
pc2.C (411–823)	−	−	−	−
RNA 3				
p3 (full length)	−	−	−	−
pc3 (full length)	+++	−	−	NT
RNA 4				
p4 (full length)	−	−	−	−
pc4 (full length)	−	−	−	−
RNA 5				
p5 (full length)	−	−	+++	+++
pc5 (full length)	−	−	−	−
RNA 6				
p6 (full length)	−	−	−	−
pc6 (full length)	−	−	−	−

[a] Amino acid positions are shown in parentheses.
[b] −, no visible colonies; +, minute colonies; +++, large colonies with strong β-galactosidase activity; NT, not tested.

To map the interacting domain in the p5 protein, the p5 gene was divided into three overlapping fragments (N, amino acids 1 to 96; M, amino acids 48 to 144; C, amino acids 97 to 191 as shown in Fig. 2) and fused with the AD-coding sequence in pACT2. When Y190 yeast cells were transformed simultaneously with pAS2-1/p5 and either pACT2/p5.N, pACT2/p5.M, or pACT2/p5.C, only yeast cells transformed with pAS2-1/p5 and pACT2/p5.N grew on synthetic medium lacking Leu, Trp, and His, and the colony lift assay showed a strong β-galactosidase activity (Fig. 2B) (2) similar to that exhibited by the intact p5-p5 interaction (Fig. 2B) (1). Y190 yeast cells transformed with pAS2-1/p5 and pACT2/p5.n (a truncated version of pACT/p5.N containing only amino acids 1 to 57) did not grow on synthetic medium lacking Leu, Trp, and His, whereas those transformed with pAS2-1/p5.N and pACT2/p5.N did grow under these conditions and exhibited strong β-galactosidase activity (Fig. 2B) (6). These results indicated that the N-terminal region (amino acids 1 to 96) of p5 contains the domain required for self-interaction. Since amino acid positions 1 to 57 in p5.n and 57 to 96 in p5.M did not interact with amino acids 1 to 96 in p5.N, there could be two subdomains required for correct conformation and interaction between two p5.N domains.

p5-p5 interaction detected by the Sos recruitment-mediated yeast two-hybrid system. The p5-p5 interaction was further examined in yeast cytoplasm by the Sos recruitment-mediated yeast two-hybrid system (1, 2). The CytoTrap two-hybrid system (Stratagene, La Jolla, Calif.) consists of a pSos plasmid expressing the human guanyl nucleotide exchange factor (hSos) as well as the *LEU2* gene, a pMyr plasmid expressing the myristylation signal peptide and the *URA3* gene, and *S. cerevisiae* strain cdc25H (15). The yeast mutant strain cdc25H has a temperature-sensitive defect in Sos, which can be rescued when the two genes of interest that are expressed as fusion proteins with hSos and the myristylation signal peptide interact such that the hSos protein is localized in the plasma membrane. Then, hSos binds Ras and activates the Ras signal transduction pathway at the nonpermissive temperature.

The RGSV p5 gene was cloned into pSos and pMyr so that p5 was expressed either as a fusion protein with hSos or fused with the myristylation signal peptide, respectively. Recombinant plasmids were prepared in *E. coli* MC1061 cells as described above. Competent cells of *S. cerevisiae* strain cdc25H were transformed with pSos/p5 and pMyr/p5 simultaneously by the lithium acetate method (7) and plated onto synthetic medium consisting of 1.5% agar plus yeast nitrogen base, with either 2% glucose or 2% galactose and containing essential amino acids and nucleotides, but lacking Leu and uracil (Ura),

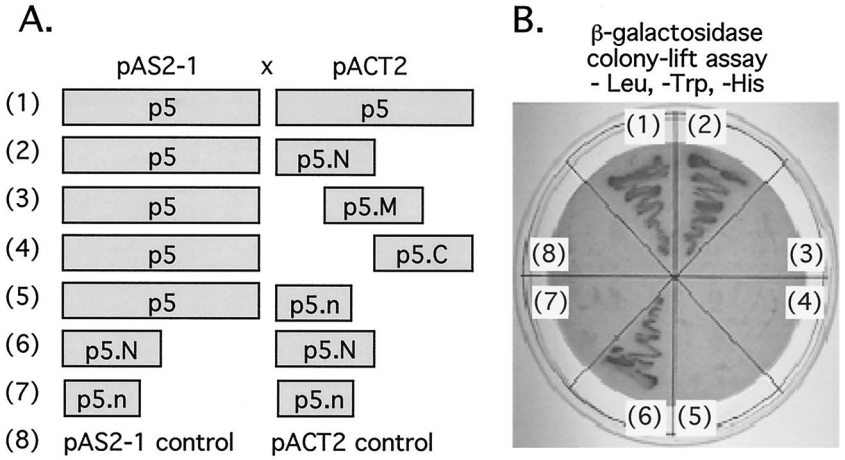

FIG. 2. Mapping of p5-p5-interacting domain. (A) Schematic presentation of p5 domains examined in the GAL4-based yeast two-hybrid assay. p5, full-length p5 (190 amino acids); p5.N, amino acids 1 to 96; p5.M, amino acids 48 to 144; p5.C, amino acids 97 to 191; p5.n, amino acids 1 to 57. (B) β-Galactosidase colony lift assay. Transformed cells were plated onto a synthetic medium plate lacking Leu, Trp, and His and incubated at 30°C for 2 days, followed by a β-galactosidase colony lift assay.

based on a protocol provided by Stratagene. First, the cotransformed yeast cells were plated on synthetic medium containing 2% glucose but lacking Leu and Ura and incubated at 25°C to allow cotransformants to form colonies (Fig. 3, lane 1). At 37°C, colonies were not formed on plates containing 2% glucose due to repression of expression of the fusion proteins (Fig. 3, lane 2). Six colonies were picked up and resuspended in 20 μl of sterile water and diluted to optical densities at 600 nm of 0.2 (lanes 3 and 7), 0.025 (lanes 4 and 8), 0.003 (lanes 5 and 9), and 0.0004 (lanes 6 and 10). Two microliters of each dilution was spotted onto synthetic medium plates containing 2% galactose, but lacking Leu and Ura, and the plates were incubated at 25 or 37°C for 2 days. cdc25H yeast cells transformed with pSos and pMyr (no inserts) and with pSos/MAFB (a transcription factor in the MAF family, type B [information about the origin not provided by the manufacturer]) and pMyr/MAFB were used as negative and positive controls, respectively. As shown in Fig. 3, cotransformants with pSos/p5 and pMyr/p5 grew vigorously at 37°C on the synthetic medium containing galactose but lacking Leu and Ura. The growth rate of the cotransformants with pSos/p5 and pMyr/p5 was much faster than that of the positive control (pSos/MAFB and pMyr/MAFB).

To examine whether p5 itself contains a membrane localization signal, cdc25H yeast cells were transformed with pSos/p5 together with pMyr containing no insert. Cotransformed cells did not grow on the synthetic medium containing 2% galactose and lacking Leu and Ura at 37°C, indicating that p5 itself does not have a signal targeted to the yeast plasma membrane to rescue the mutant yeast Ras signal transduction pathway at the nonpermissive temperature. This result confirmed that the p5-p5 interaction occurred in the cytoplasm of the yeast cells cotransformed with pSos/p5 and pMyr/p5.

Far-Western blots of p5-p5 interaction. As described above, RGSV p5 was shown to interact with itself both in the nucleus and in the cytoplasm of yeast by two different yeast two-hybrid systems. The interaction was further examined in vitro by far-Western blots. As a probe for these blots, p5.H (p5 with six His residues at the C terminus) was prepared by using the

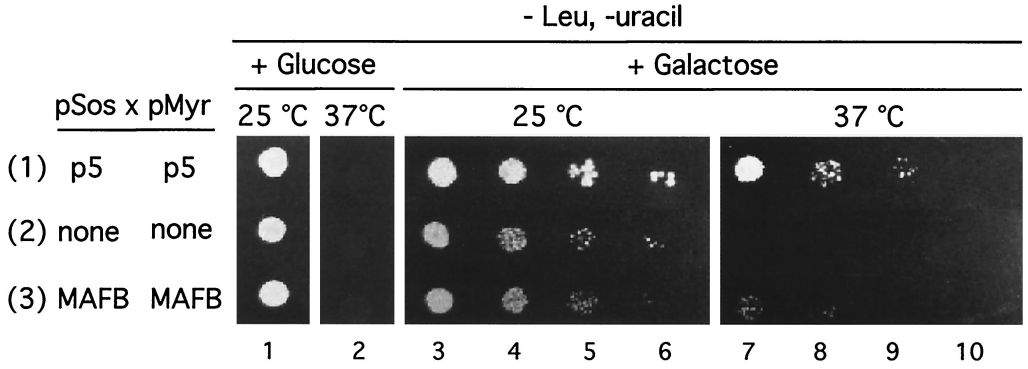

FIG. 3. Detection of p5-p5 interaction by the Sos recruitment assay. *S. cerevisiae* strain cdc25H was transformed with pSos/p5 and pMyr/p5 (row 1), pSos and pMyr as a negative control (row 2), and pSos/MAFB and pMyr/MAFB as a positive control (row 3). Transformed cells were first grown at 25°C on synthetic medium containing 2% glucose, but lacking Leu and Ura (lane 1) or at 37°C (lane 2). Serial dilutions of yeast cell suspensions were spotted onto synthetic medium plates containing 2% galactose, but lacking Leu and Ura, and incubated at 25°C (lanes 3, 4, 5, and 6) or 37°C (lanes 7, 8, 9, and 10) for 2 days.

Sagiyama Alphavirus transient expression vector (18) in cultured BHK21 cells as follows. The p5 gene followed by six-His codons and a TGA termination codon was inserted in-frame downstream of the N-terminally-deleted capsid protein gene in the place of the GFP.H gene in pSAG2.ΔC:GFP.H (18), designated pSAG2.ΔC:p5.H. In vitro transcripts from pSAG2.ΔC:p5.H and pSAG2.3L (18), which is a helper construct providing all of the structural proteins for pseudovirion formation, were electroporated into BHK21 cells as described previously (18). After incubation of electroporated cells at 30°C for 2 days, two types of pseudovirions—one containing the p5-expressing replicon RNA and another containing structural protein-expressing helper RNA—were passaged again in BHK21 cells at 30°C for 5 days to increase the titer of replicon RNA-containing pseudovirions. This mixture of pseudovirions was then used to inoculate BHK21 cells, which were incubated at 30°C for 2 days for expression of p5.H in the cytoplasm. Infected BHK21 cells were lysed in 1% NP-40, and p5.H was purified with Ni-nitrilotriacetic acid (NTA) agarose resin (QIAGEN) according to the manufacturer's protocol.

The 48-kDa glutathione-*S*-transferase (GST)-p5 fusion protein, which had been prepared for generation of anti-p5 antiserum (5); the 26-kDa GST protein expressed from pGEX6P-1 (Amersham Pharmacia) and purified with glutathione Sepharose 4B resin as recommended by the manufacturer, and the 26-kDa dihydrofolate reductase (DHFR) tagged with six His residues at the C terminus expressed from pQE16 (QIAGEN) and purified with Ni-NTA resin based on the manufacturer's protocol, were run at approximately 1 μg per lane in two sets of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE [12.5% polyacrylamide]) gels (11). Figure 4A shows the protein pattern after staining the gel with Coomassie brilliant blue. Another gel was blotted onto a nitrocellulose membrane. After blocking the blotted membrane in TBSN buffer (20 mM Tris-HCl [pH 7.5], 0.15 M NaCl, 0.1% NP-40) overnight, the membrane was probed with p5.H at 10 mg/ml in TBSN overnight.

After being probed with p5.H, the membrane was extensively washed in TBSN and treated with anti-six-His antibody conjugated with an alkaline phosphatase (Invitrogen), followed by washing in TBSN. Finally, the membrane was immersed in a substrate solution containing 5-bromo-4-chloro-3-indryl phosphate and nitroblue tetrazolium in a mixture of 10 mM Tris-HCl (pH 9.5), 0.1 M NaCl, and 5 mM $MgCl_2$ until color development was saturated. As shown in Fig. 4B, the GST:p5 band was clearly detected (lane 1), but GST itself was not detectable (lane 2). Color development with DHFR.H (positive control) in lane 3 was very strong, because the protein sample itself contained a six-His tag at the C terminus. This result confirmed that the p5-p5 interaction occurs in vitro as well as in yeast nucleus and cytoplasm.

p5 forms a large complex in RGSV-infected rice cells. We examined formation of a multimeric p5 complex in infected rice tissue by sucrose density gradient centrifugation of protein extracts, followed by native PAGE and Western blot analysis. Five grams of RGSV-infected rice leaves was ground to powder in liquid nitrogen in a mortar and pestle and homogenized in a mixture containing 5 ml of 0.1 M Tris-HCl (pH 7.5), 10 mM KCl, 5 mM $MgCl_2$, 5% sucrose, and 10 mM 2-mercaptoethanol. The homogenate was filtered through four layers of gauze, and the filtrate was centrifuged at 10,000 rpm for 10 min in a Beckman JA-25.50 rotor. One milliliter of the supernatant was layered onto a sucrose density gradient in an SW41 tube (11 ml of 10 to 40% sucrose in grinding buffer) and centrifuged at 38,000 rpm for 12 h at 4°C. The gradient was fractionated into 20 fractions of 0.6 ml each. Fraction 20 was used for resuspension of the pellet. Five microliters from each fraction was mixed with 5 μl of 2× sample buffer (0.1 M Tris-HCl [pH 9.0], 4% SDS, 30% sucrose, 10% 2-mercaptoethanol) and run on an SDS-PAGE (12.5% polyacrylamide) gel (11). A gel stained with Coomassie brilliant blue (Fig. 5A) showed that fraction 10 contained the majority of the ribulose bisphosphate carboxylase/oxygenase complex (Rubisco), an oligomeric complex of ~560 kDa composed of multiple copies of both a 53-kDa large subunit and a 14.9-kDa small subunit. These 20 samples were also run on four other SDS-PAGE (12.5% polyacrylamide) gels, which were separately subjected to Western blot analysis with antisera against p2 (5), p5 (5), p6 (14), and RNP particles (9) to identify fractions containing the p2, p5, p6, and N proteins. Fractions 3, 4, and 5 contained the most p5, and fractions 2, 3, and 4 contained the most p4, whereas N was found primarily in fraction 20, the bottom fraction, presumably in RNP particles (data not shown). p2 was clearly detected from fractions 3, 4, and 5. Fraction 5 (containing p2, p5, and p6) and fraction 10 (containing Rubisco) were each dialyzed against sucrose-free grinding buffer, and 5 μl of each was run on a 5% native PAGE gel. Proteins were transferred to a nitrocellulose membrane and probed with anti-p2, anti-p5, or anti-p6 antisera prepared in rabbits (5, 9), followed by treat-

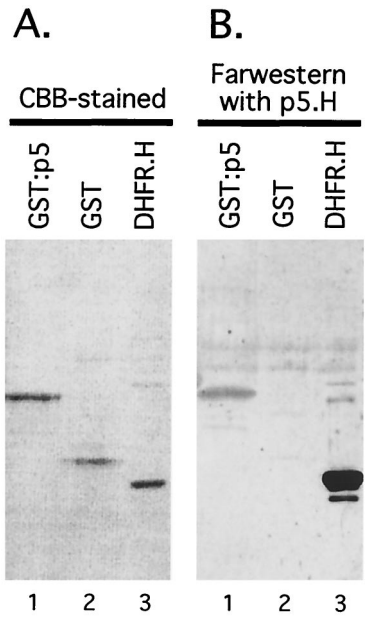

FIG. 4. Far-Western analysis of the p5-p5 interaction. One microgram each of GST:p5 (lane 1), GST (lane 2), and DHFR.H (lane 3) was run on two SDS-PAGE (12.5% polyacrylamide) gels. (A) One gel stained with Coomassie brilliant blue. (B) Proteins in the other gel were blotted onto a nitrocellulose membrane and probed with p5.H, followed by detection of the six-His tag by anti-His tag antibody conjugated with alkaline phosphatase. 5-Bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium were used as substrates for colorimetric detection.

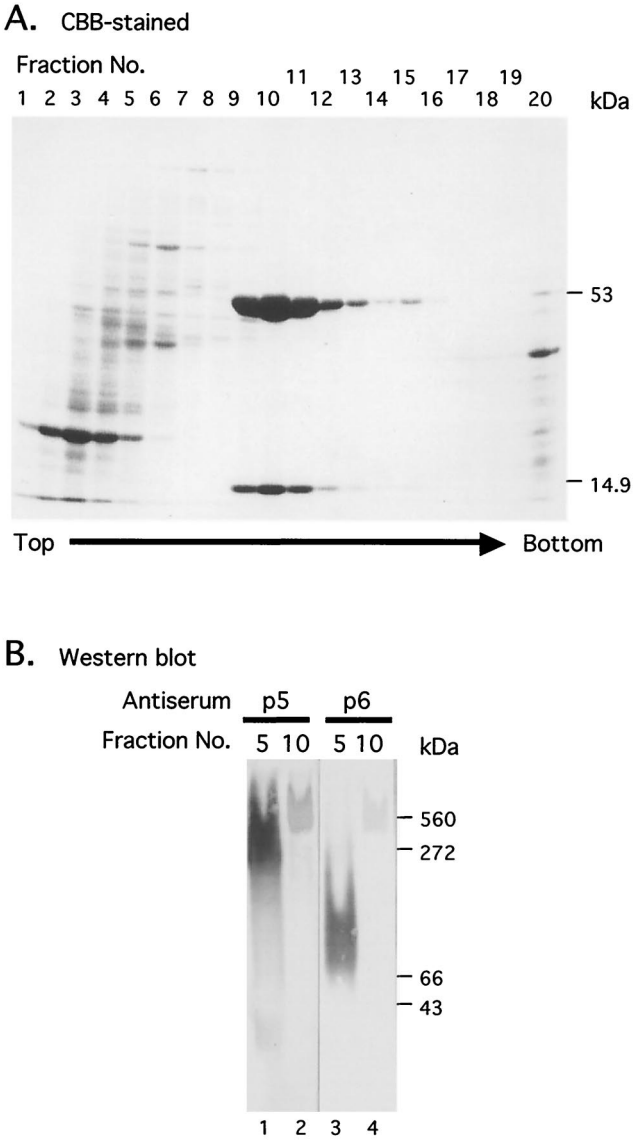

FIG. 5. Detection of native p5 and p6 proteins in extracts from RGSV-infected rice leaf tissues fractionated by sucrose density gradient centrifugation. (A) Coomassie brilliant blue-stained gel. RGSV-infected rice leaf extracts were loaded on a 10 to 40% sucrose density gradient, which was spun at 38,000 rpm for 12 h in an SW 41 rotor and fractionated into 20 fractions from the top to the bottom. A 5-μl aliquot from each fraction was run on an SDS-PAGE (12.5% polyacrylamide) gel, which was stained with Coomassie brilliant blue. The positions of the large and small subunits of Rubisco (53 and 14.9 kDa, respectively) are shown to the right. (B) Western blot. Proteins in fractions 5 and 10 were run on a 5% native PAGE gel, which was blotted onto a nitrocellulose membrane. Proteins on the membrane were detected by anti-p5 antiserum (lanes 1 and 2) or anti-p6 antiserum (lanes 3 and 4). The positions of native protein size markers are shown on the right. Rubisco, 560 kDa; urease trimer, 272 kDa; bovine serum albumin, 66 kDa; ovalbumin, 43 kDa.

ment with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G. After washing the membrane, proteins were detected by adding 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium as substrates. An intense band was detected with anti-p5 antiserum at the position of about 260 kDa (Fig. 5B, lane 1) by comparison with Coomassie brilliant blue-stained size markers (not shown). p6 was found at ~130 kDa, which is a different position from that of p5 (Fig. 5B, lane 3), indicating that p5 and p6 are components of different complexes. p2 could not be detected as a distinct band, but appeared as a weak smear (data not shown). Based on the mass of the complexes, p5 may be present as a 12-mer, whereas p6 may form a hexamer. Alternatively, p5 and p6 could form heterocomplexes with unidentified host proteins.

Results from a previous study (5) and from this study showed that the RGSV p5 protein is expressed both in RGSV-infected rice leaves and viruliferous brown planthoppers and interacts with itself through its N-terminal domain, but does not interact with other RGSV proteins in a GAL4-based yeast two-hybrid experiment. The p5-p5 interaction was confirmed to occur in the yeast cytoplasm and thus probably takes place in rice plant cells as well. p5 protein was found as a soluble ~260-kDa protein complex in an extract from RGSV-infected rice cells. The ~260-kDa protein complex did not contain either p6 or p2. Further experiments to screen for host factors that interact with p5 and other RGSV proteins are in progress, and these results should clarify the role of functional complexes in RGSV replication.

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