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Detection of Rice Tungro Bacilliform Virus Gene Products *in Vivo*

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To study the products of the open reading frames (ORFs) of rice tungro bacilliform virus in rice plants the sequences containing ORFs I (encoding a 24-kDa protein, P24) and IV (P46) and the protease and polymerase (reverse transcriptase + RNaseH) domains of ORF III were cloned into a pGEX expression vector. The proteins, which were C-terminal fusions to glutathione *S*-transferase, were expressed in *Escherichia coli* and antisera were raised against them which, together with an antiserum against virus particles, was used to probe blots of proteins from infected and uninoculated plants and from virus preparations. The P24 antiserum detected virus-specific proteins of 74, 60, and 52 kDa, which are much bigger than expected. These proteins were found in virus preparations and immunogold labeling suggested that they might be internal in the particles. Virus-specific proteins of 33, 37, 62, and >150 kDa were revealed by antiserum to virus particles. The antiserum to the protease revealed proteins of 13.5, 37, and 68 kDa both in extracts from infected plants and in purified virus preparations. This antiserum decorated intact virus particles as did the particle antiserum. The polymerase domain antiserum reacted with products of 56, 65, and 68 kDa in extracts from infected plants but not in virus particles. The antiserum to the ORF IV product did not detect any bands in either infected plant extracts or virus preparations. The significance of these products is discussed.

INTRODUCTION

Rice tungro disease is reported from at least 10 countries in South and Southeast Asia (Hibino, 1987), causing an annual loss in excess of $\$1.5 \times 10^9$ (Herdt, 1991). The disease is caused by a complex of two viruses, rice tungro spherical virus (RTSV) and rice tungro bacilliform virus (RTBV) (Hibino *et al.*, 1978; Saito *et al.*, 1976). RTSV is an RNA virus which is transmitted independently by a leafhopper vector (*Nephotettix virescens*). RTBV is a DNA virus and is responsible for symptoms such as plant stunting and yellow leaf discoloration, but depends on RTSV for transmission by the vector. Together the viruses cause severe "tungro" symptoms and result in reduced yield and crop losses (Hibino *et al.*, 1978).

Molecular analyses of the RTBV genome indicate it belongs to the badnavirus group (Lockhart, 1990), the type member being *Commelina* yellow mottle virus (CoYMV) (Medberry *et al.*, 1990). RTBV has a circular double-stranded DNA genome which is interrupted by two discontinuities (Jones *et al.*, 1991). The positions of the discontinuities have been mapped to specific sites and are postulated as the priming sites for DNA replication (Bao and Hull, 1992), with the virus replicating in a manner analogous to that of caulimoviruses (Covey, 1985; Mason *et al.*, 1987). The complete sequences of two isolates and the partial sequence of a third isolate of RTBV from the Philippines have been published (Hay *et al.*, 1991; Qu *et al.*, 1991; Kano *et al.*, 1992). The full sequence reported by Hay *et al.* (1991) was of an infectious clone (Dasgupta *et al.*, 1991). The two full sequences show the genome is approximately 8.0 kbp and that one strand potentially contains four large open reading frames (ORFs) (Fig. 1). The first ORF (ORF I) is capable of encoding a protein of 24 kDa (P24), but has no initiation (AUG) codon. However, some evidence suggests that protein synthesis could be initiated at an AUU codon (J. Fütterer and T. Hohn, personal communication). The other three ORFs (ORFs II–IV) potentially encode proteins of 12, 194, and 46 kDa (P12, P194, and P46). The functions of P12, 24, and 46 are unknown. Comparative analyses with the three sequenced badnaviruses, CoYMV, cocoa swollen shoot virus, and sugarcane bacilliform virus (Medberry *et al.*, 1990; Bouhida *et al.*, 1993; Hagen *et al.*, 1993), and with the caulimoviruses (Franck *et al.*, 1980; Hasegawa *et al.*, 1989; Richins *et al.*, 1987; Hull *et al.*, 1986) suggest that P194 is a polyprotein that is proteolytically cleaved to yield the virion coat protein (indicated by the RNA binding site consensus sequence), an aspartic protease and replicase (reverse transcriptase (RT) and RNase H) characteristic of retroelements.

Here we describe the expression of ORFs I and IV and of the protease and replicase domains of ORF III as fusion proteins with glutathione *S*-transferase (GST) in *Escherichia coli* using the pGEX vector. Antisera to these

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TABLE 1

OLIGONUCLEOTIDE PRIMERS USED TO GENERATE PCR PRODUCTS ENCODING RTBV ORFS P24, P46 AND THE REVERSE TRANSCRIPTASE AND PROTEASE REGIONS OF P194

ORF	Primer name	Primer[a]	Nucleotide position	PCR product (bp)
P24	V300	5′-gtcgacggatcc GTT CCA AAG AGG GAT CTT ATT TC-3′ (*Bam*HI)	69–91, plus strand	599
	V301	5′-gagctccccggg TCA TGT AGC TTG ATG CTT AAG-3′ (*Sma*I) *	648–668, minus strand	
Protease	V354	5′-aagcttgtcgacggatc CTA GCA CTA GAA GTG ACA-3′ (*Bam*HI)	3634–3651, plus strand	1169
	V280	5′-tctagaccccgggaattc TTC ATG TCT GGC TGT TGG-3′ (*Eco*RI)	4270–4287, minus strand	
RT/RH	V776	5′-gggacgtctagaggatccatg gCT ACG GGA ATA TAC CCA ACA GCC-3′ (*Bam*H)	4256–4278, plus strand	651
	V774	5′-ggggatccggccggtaccgaattc TTA GGC ACT CTT TTC CTT TAG CC3′ (*Eco*RI) *	5999–6021, minus strand	
P46	V329	5′-tctagacccggg ATG AAT ATA GAG TAC CCG-3′ (*Sma*I)	6042–6059, plus strand	1765
	V330	5′-gaattcgcatgc TTA AGC ATT GTC CAT ACG-3′ (*Eco*RI) *	7194–7211, minus strand	

[a] Restriction enzyme (RE) sequences are given in lowercase letters: nucleotides complementary to the viral sequence are given in uppercase letters; the RE sites used for cloning into pGex-3X are underlined; *indicates stop codons.

fusion proteins have been raised and studies to detect gene products in RTBV-infected rice are presented.

MATERIALS AND METHODS

PCR of sequences encoding potential gene products

To isolate and clone the coding region of each ORF, 10 ng of pJIIS2, encoding the full sequence of RTBV (Jones *et al.*, 1991), was linearized with either *Sal*I or *Bam*HI and incubated with equimolar amounts (0.25 μmol) of the relevant forward and reverse primers in a 100-μl reaction mixture containing 1.5 m*M* $MgCl_2$, 50 m*M* KCl, 200 μ*M* dATP, dTTP, dGTP, and dCTP each, and 2.5 U Taq polymerase (Boehringer-Mannheim) in 10 m*M* Tris–HCl, pH 8.4, buffer. Each reaction was overlaid with DNase/RNase-free mineral oil (Sigma). The polymerase chain reaction (PCR) was performed automatically using a Thermal Controlling Unit (Techne, PH-3) programmed for 25 cycles, each cycle including denaturation for 1 min at 93°, annealing at 50° for 2 min, and polymerization at 72° for 3 min. Following completion of the PCR, the sample was extracted twice with an equal volume of phenol and the DNA product concentrated by ethanol precipitation by standard procedures (Sambrook *et al.*, 1989). The dried pellet was resuspended in 10 μl of sterile H_2O and a 1-μl sample was analyzed in a 1% agarose gel in 1× TBE (100 m*M* Tris–borate, 10 m*M* EDTA, pH 8.0) to determine the size and quantity of the PCR products.

Forward and reverse primers (Table 1 and Fig. 1) flanking ORFs I and IV and flanking the putative protease and reverse transcriptase functions encoded on ORF III were chosen from the published RTBV sequence (Hay *et al.*, 1991) and synthesized using a Gene Assembler Plus (Pharmacia). Each primer contained either a 5′ or 3′ nonviral extension of 6–18 nucleotides encoding specific restriction enzyme recognition sequences to facilitate cloning.

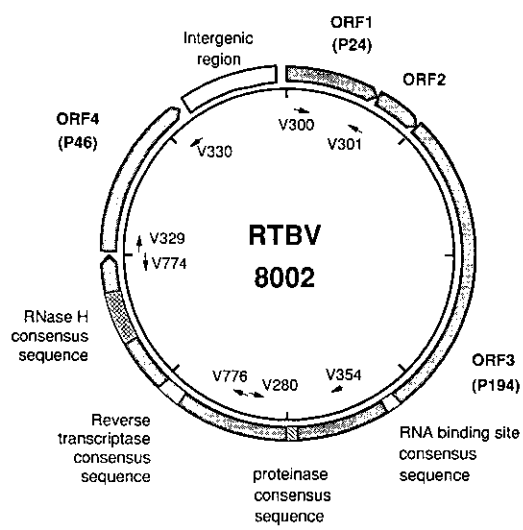

FIG. 1. Map of RTBV DNA showing positions of primers used to generate PCR products encoding P24 and P46 and the reverse transcriptase and protease regions of P194. The ORFs identified in Hay *et al.* (1991) are indicated. The location of consensus sequences of the RNA binding site (located within the coat protein), aspartate protease, reverse transcriptase, and RNase H within P194 are shown.

Cloning into expression vector

PCR products were digested with the appropriate restriction enzymes, directionally cloned into the expression vector pGEX-3X (Pharmacia), and transformed into *E. coli* strain JM83 following standard procedures (Sambrook *et al.*, 1989). Recombinant clones were sequenced by the dideoxy-termination method (Sanger *et al.*, 1977) using a Sequenase Version 2.0 kit (U.S. Biochemical Corp.) and sequencing primers, V402 5′-GTATTGAAG-CTATCCCAC-3′ (nucleotides 802–819, plus strand) and V313 5′-AGCTGCATGTGTCAGAGG-3′ (nucleotides 1082–1099, minus strand) complementary to pGEX-3X sequences flanking the cloning domain. When necessary, additional primers hybridizing to internal regions of the insert were used to complete sequencing both strands.

Expression and purification of fusion proteins

Fusion proteins were expressed using the modified procedure described by Grieco *et al.* (1992). Briefly, 1 liter of *E. coli* containing a recombinant pGEX plasmid was induced with 0.1 m*M* isopropyl β-thiogalactopyranoside for 4 hr. The cells were pelleted by centrifugation, resuspended in phosphate-buffered saline (PBS) and lysed by sonication. The lysate was centrifuged at 10,000 *g* for 10 min and the supernatant held on ice. The pellet was resuspended in 10 ml of PBS containing 25 m*M* triethanolamine, 1.5% sarkosyl (Sigma), and 1 m*M* EDTA, pH 8.0, and agitated gently for 10 min at 4°. Triton X-100 and $CaCl_2$ were added to a final concentration of 2% and 1 m*M*, respectively, and the mixture was centrifuged at 10,000 *g* for 10 min. The supernatant was pooled with the first supernatant and the fusion protein was purified through a glutathione–Sepharose 4B column (Pharmacia) according to the supplier's recommendations. The fusion proteins were diluted in SDS sample buffer and electrophoresed in a 12% polyacrylamide gel (Laemmli, 1970). The pGEX-3X vectors have been engineered so that the GST carrier can be cleaved from fusion proteins by digestion with the site-specific protease, factor Xa. In order to verify the purification of the fusion proteins, the protein was cleaved with factor Xa and the cleavage products analyzed in a 12% polyacrylamide gel. Forty micrograms of protein was dialyzed against 100 vol of factor Xa buffer (20 m*M* Tris–HCl, pH 8.0, 100 m*M* NaCl, 2 m*M* $CaCl_2$, 1 m*M* sodium azide) for 4 hr and incubated with 1 μg of factor Xa (New England Biolabs) overnight at 25°.

Production of antisera

The recovery of RTBV proteins cleaved from the GST carrier by factor Xa digestion was consistently low and therefore the GST–ORF fusions rather than liberated RTBV proteins were used to immunize rabbits and raise antisera. Fusion proteins were purified as described and separated in a 12% polyacrylamide gel. The gel was stained with 0.25% Coomassie brilliant blue R-250 in 5% acetic acid, 5% methanol to reveal the fusion protein and the band was excised from the gel. The gel slice was rinsed briefly in 1× running buffer (25 m*M* Tris, 250 m*M* glycine, 0.1% SDS) and the protein eluted from the acrylamide by dialyzing the gel slice against 1× running buffer at room temperature for several hours. The protein was dialyzed against water overnight and was concentrated by lyophilization. Approximately 100 μg of protein was resuspended in 750 μl of PBS and emulsified with an equal volume of complete Freund's adjuvant before being subcutaneously injected into each of two rabbits. A booster injection of protein emulsified with incomplete Freund's adjuvant was given after 2 weeks; after a further 2 weeks each rabbit was bled at weekly intervals until the titer of the sera fell, as shown by Western blot analysis. The crude serum was routinely used at 1:1000 dilution for all Western analyses. To absorb any antibodies reacting against uninoculated plant proteins, 50 μl antiserum was added to 10 mg of an acetone powder of uninoculated rice leaves which had been suspended in 500 μl PBS. After incubating at 37° for 1 hr and at 4° for 18 hr with shaking, the acetone powder was removed by centrifugation.

Protein analysis

All samples were analyzed by electrophoresis in 12% polyacrylamide gels as described by Laemmli (1970). Viral proteins were extracted from 0.5 g of infected leaf tissue in 1.0 ml of grinding buffer (100 m*M* Tris–HCl, pH 7.5, 10 m*M* KCl, 5 m*M* $MgCl_2$, 0.4 *M* sucrose, 10% glycerol, 10 m*M* 2-mercaptoethanol). The extract was microfuged for 10 min and 10- to 20-μl aliquots of the supernatant were fractionated on an SDS–polyacrylamide gel. The pellet was reextracted in 1.0 ml of urea buffer (4.5% (w/v) SDS, 9 *M* urea, 1.1 *M* 2-mercaptoethanol, 0.05% bromophenol blue, 75 m*M* Tris–HCl, pH 6.8), boiled for 10 min and clarified by centrifugation for 10 min. Ten to 20 ml of the supernatant was loaded directly onto SDS–polyacrylamide gels. Approximately 500 ng RTBV, purified from leaf tissue insect-infected with the Philippine isolate as described by Jones *et al.* (1991), was loaded. Following electrophoresis, the fractionated proteins were transferred onto a nitrocellulose filter (Hybond C, Amersham) using a semidry electroblotter and immunogenic proteins were detected (Towbin *et al.*, 1979) with either chromogenic or enhanced chemiluminescence (ECL, Amersham) visualization. The chromogenic method used an alkaline phosphatase goat anti-rabbit IgG (Sigma) at a 1:9000 dilution, and proteins were visualized in the presence of nitroblue tetrazolium and 5-bromo-4-chloro-3-indoyl phosphate. For ECL, immunogenic proteins were probed with antibodies conjugated to horseradish peroxidase and were visualized in the presence of luminol and hydrogen peroxidase.

Electron microscopy

For direct immunogold staining with the protease and coat protein antisera 3–5 μl of a virus preparation was air dried onto pyroxilin (4%)-coated gold grids previously treated with one drop of Butvar solution and incubated on 10 μl of antiserum diluted 1:10 in blocking buffer (10 m*M* Tris–HCl, pH 7.4, 0.9% NaCl, 0.05% PEG 2000, 3% BSA) for 1 hr at room temperature (Beesley *et al.,* 1985). The grids were washed four times in 10 μl of blocking buffer for 1 min and blotted dry. They were then floated on 10 μl of goat anti-rabbit serum conjugated with 15-nm gold particles (Sigma) diluted 1:20 in blocking buffer for 1 hr at room temperature. The grids were washed four times in 10 μl of distilled water for 1 min and stained with 5 μl uranyl acetate for 5 min before viewing under a Joel 1200 electron microscope.

For the experiments with the P24 antiserum it was necessary to capture virus particles first using antiserum (immunecapture). Grids were incubated at room temperature for 1 hr on a 10-μl drop of coat protein antiserum diluted 1:1 with water and then washed with blocking buffer. They were then placed for 1 hr at room temperature on drops of crude virus preparations and then washed again with blocking buffer. Denaturation of the immunecaptured particles was by incubation of the grids with a 1/200 dilution of Decon 90 (Decon Laboratories Ltd.) in water for 10 min at room temperature followed by washing the grids several times with blocking buffer. IgG was prepared from P24 antiserum as described by van Regenmortel (1982) and was conjugated with 15-nm colloidal gold according to the manufacturer's (British Biocell International) instructions. The immunecaptured particles were reacted with a 1/50 dilution of the gold-conjugated P24 antiserum and observed in the electron microscope as described above.

RESULTS AND DISCUSSION

Expression and purification of the fusion proteins

The expression vector pGEX-3X directs the synthesis of foreign polypeptides as fusions with a 27-kDa GST (Smith and Johnson, 1988). The GST forms an amino-terminal affinity tail on the fusion protein and facilitates purification by affinity chromatography onto immobilized glutathione under denaturing conditions. RTBV sequences encoding ORFs I (P24) and IV (P46) and the putative protease and replicase domains of ORF III (Pprot and PRT) were generated by PCR and were cloned into the multiple cloning sites in pGEX-3X. The recombinant clones were expressed but following cell lysis none of the fusion proteins were soluble in aqueous solution. The inclusion of sarkosyl as a modification to the original protocol for purifying GST-fused proteins as described by Grieco *et al.* (1992) allowed the solubilization and recovery of the recombinant proteins. For each construct, approximately 2–4 mg of the fusion proteins were recovered from 1 liter of bacterial culture, each protein being approximately the expected size (Table 2).

TABLE 2

PRODUCTS OF pGEX EXPRESSION OF RTBV PROTEINS

RTBV protein	Size (kDa)	
	Fusion protein	Factor Xa cut protein
P24	50	27,[a] 24
Pprot	38	27,[a] 11
PRT	92	27,[a] 66
P46	72	27,[a] 46

[a] Expected size for GST.

The pGEX vector carries the recognition sequence for a protease, factor Xa, between the GST and the multiple cloning site and so the GST carrier can be cleaved from the fusion protein. To confirm the expression of the RTBV protein fusions, they were incubated with factor Xa and two polypeptides, 27 kDa (GST) and the relevant RTBV protein were liberated (Table 2). The RTBV proteins were purified from the GST carrier and the remaining uncleaved fusion protein by absorption of the GST on glutathione–Sepharose columns. The recovery of the cleaved RTBV proteins, however, was consistently low and so the fusion proteins rather than the liberated RTBV proteins were used to immunize rabbits and raise antisera.

Detection of RTBV gene products

Proteins were extracted from RTBV-infected rice plants and separated into soluble and insoluble fractions as described under Materials and Methods. These proteins, along with samples from virus preparations, were separated in 12% polyacrylamide gels and blotted onto nitrocellulose membranes. The membranes were analyzed by immunological detection with antisera raised against P24, P46, Pprot, and PRT by conventional Western blotting detection procedures or by the more sensitive ECL detection procedure. Proteins extracted from uninoculated rice plants were used as controls. All antisera were cross-absorbed against uninoculated plant proteins before use. Because all the antisera were raised against fusions with GST, some of them were also cross-absorbed against expressed GST protein. No observable difference was detected on identical blots probed with antisera with or without cross-absorption against GST, indicating there were no GST antigens present in infected or healthy plants (data not presented). All blots were also probed in parallel with the relevant preimmune sera collected from rabbits before immunization, which had been cross-absorbed with uninoculated plant extract. No proteins were detected with these control sera

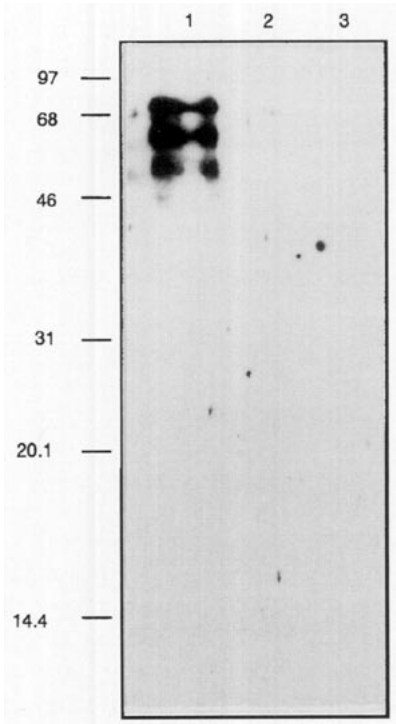

FIG. 2. ECL Western blot probed with antiserum raised against P24. Lane 1, RTBV particles; lane 2, soluble proteins isolated from uninfected rice plants; lane 3, soluble proteins isolated from RTBV-infected rice plants. The ECL molecular weight markers (kDa) (Amersham) were applied as directed by the manufacturer.

(data not presented). None of the antisera detected proteins in the insoluble fraction prepared from either uninoculated or infected plants (data not presented).

P24

Figure 2 shows an ECL blot probed with P24 antiserum. Proteins of apparent molecular weights 74, 60, and 52 kDa were specifically detected in purified virus particles (Fig. 2, lane 1), but no proteins were detected in the soluble extracts from either uninoculated or infected rice plants (Fig. 2, lanes 2 and 3). The lack of detection in infected plant extracts is likely to be due to the proteins being present in much lower concentration than in purified virus preparations. The proteins detected in purified virus preparations were much larger than expected but were not obvious multimers of P24. Since virus preparations were from plants jointly infected with RTBV and RTSV there was a possibility that RTSV proteins were being detected. However, when blots of coat proteins from purified RTSV were probed with the P24 antiserum there was no signal (data not presented). The nature of these proteins is currently being investigated.

As noted in the Introduction, ORF I of RTBV, which encodes P24, does not contain an AUG start codon (Hay *et al.*, 1991). The detection of products which react with P24 antiserum indicates that this ORF is expressed in infected plants though probably at low levels.

The finding of P24 in virus preparations was unexpected. To further investigate this, virus preparations were reacted with P24 antiserum conjugated with gold particles, negatively stained, and viewed under an electron microscope. P24 antiserum did not label intact particles (Fig. 3A) but there was some label on small clumps of material. To determine if this was degraded RTBV, particles which were captured on grids precoated with particle antiserum as described under Materials and Methods were then treated for 10 min with a 1/200 dilution of Decon 90. When these grids were probed with gold-labeled P24 antiserum it was found that clumps of material thought to be degraded virus particles were labeled (Fig. 3B). Controls of sap from noninoculated plants either untreated or treated with Decon 90 did not show any labeling. This suggests that the antigen could be within the virus particles and therefore might be associated with particle assembly.

RTBV particle antiserum

Jones *et al.* (1991) and Qu *et al.* (1991) reported two proteins, 33 and 37 kDa, from RTBV particles. As only the 37-kDa protein was found in extracts from infected plants it was suggested that the 33-kDa protein was processed from it (Qu *et al.*, 1991).

Antiserum raised against intact RTBV particles was kindly provided by Dr. Hibino of the International Rice Research Institute (Los Banos, Philippines). Both chromogenic (Fig. 4) and ECL visualization revealed proteins of 33, 37, 62, and sometimes >150 kDa in both virus preparations and extracts of infected sap; there were also several lower molecular weight bands in virus preparations (Fig. 4) which possibly reflect degraded coat protein products. The relative proportions of these proteins varied from sample to sample but, in general, there was more 37- than 33-kDa protein in extracts from infected plants than in purified virus preparations. No proteins were observed in sap from noninoculated plants.

The several protein species revealed by the virus particle antiserum were in the supernatant fraction, but not in the pellet fraction, from low speed centrifugation of extracts from infected plants. This differs from the results of similar experiments on cauliflower mosaic virus where, because the virus particles are in inclusion bodies, they sediment at low speed (Hull and Shepherd, 1976) and support the electron microscopic evidence that RTBV particles are not associated with inclusion bodies (Favali *et al.*, 1975; Sta. Cruz and Koganezawa, 1991).

Protease

The region of the P194 used to express the protein to raise antiserum against the aspartate protease was defined by modeling the RTBV amino acid sequence against that of the aspartate protease of human immunodeficiency virus-1 (HIV1) (R. Hull and A. Wilderspin, un-

published observation). Western blots probed with this antiserum revealed a major protein of approximately 13.5 kDa in virus preparations (Figs. 5A, lane 1 and 4B). The size of 13.5 kDa is that expected from the modeling of the RTBV sequence against HIV1 aspartate protease. Minor proteins of higher molecular weight were also detected by ECL in the virus preparation (37 kDa) (Fig. 5A, lane 1) and in infected sap (68 kDa) (Fig. 5A, lane 3), as well as the 13.5-kDa protein; these were not revealed by the chromogenic method (Fig. 5B). No proteins were detected in extracts from healthy plants (Fig. 5A, lane 2).

As the antiserum detected the protease in RTBV preparations, virus particles were immunogold labeled with Pprot antiserum (Fig. 3C). This clearly showed the label attached to intact virus particles as did labeling with particle antiserum (Fig. 3D).

PRT

The RT/RNaseH domain of P194 was defined as that C-terminal to the putative aspartate protease and an antiserum was raised against the expressed protein from that region. On Western blots with that antiserum no protein bands were visualized by chromogenic detection, but ECL analysis revealed three major proteins of 56, 65, and 68 kDa and two minor proteins of 25 and 30 kDa in

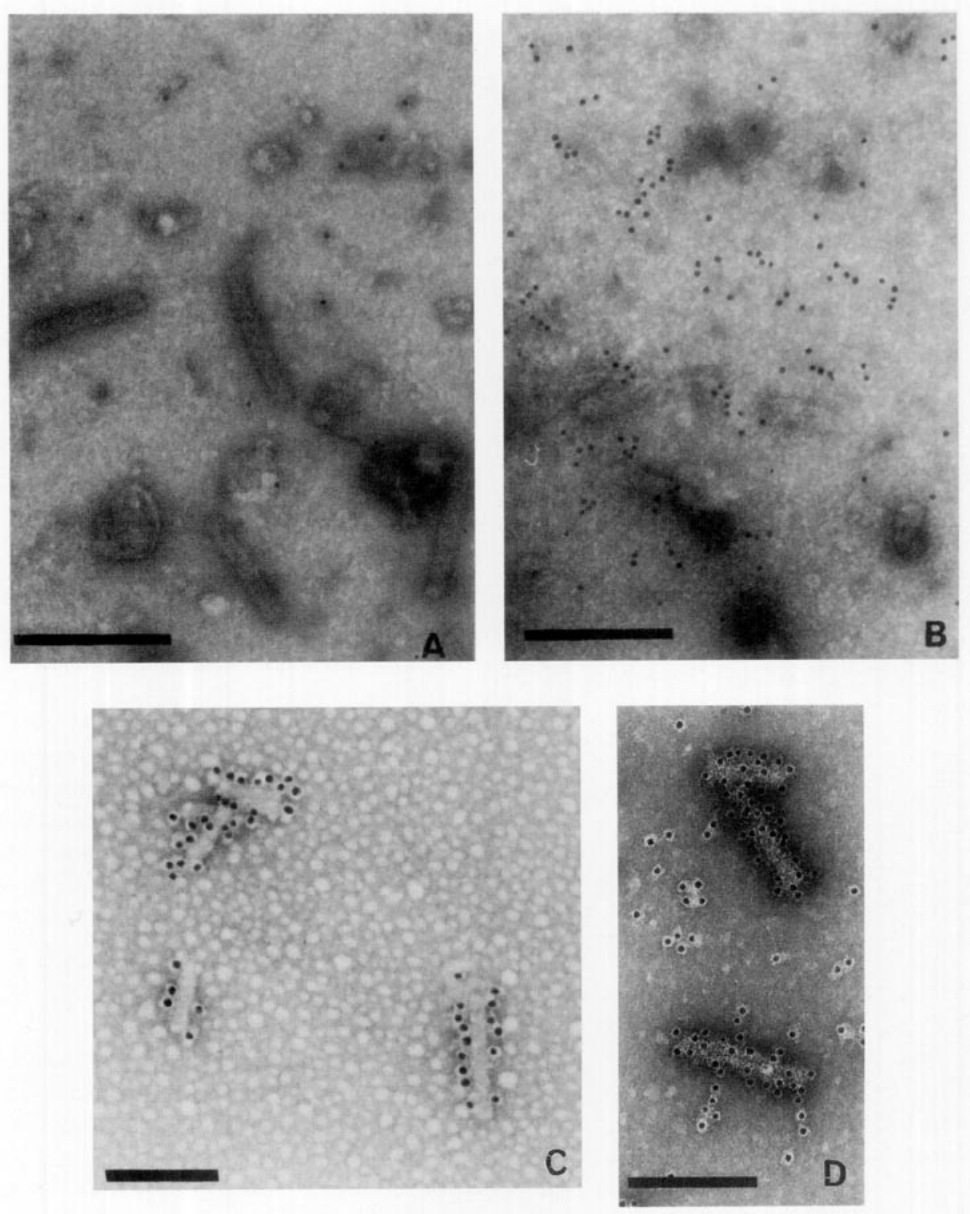

FIG. 3. Electron micrographs of RTBV immunogold labeled with various antibodies. (A) Intact particles immunecaptured with coat protein antiserum and then reacted with gold-labeled P24 antiserum. (B) Particles immunecaptured with coat protein antiserum, degraded with Decon 90, and probed with gold-labeled P24 antiserum. (C) Particles gold labeled with protease antiserum. (D) Particles gold labeled with coat protein antiserum. Bar, 200 nm.

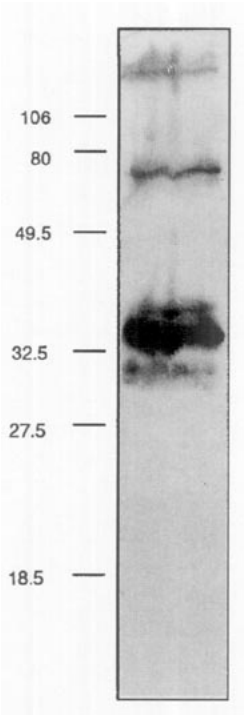

FIG. 4. Western blot of RTBV particle proteins probed with antiserum raised against intact RTBV particles and detected by the chromogenic method. Prestained protein markers (Gibco) were applied as directed by the manufacturers.

the soluble fraction of infected tissue (Fig. 6, lane 3). No proteins were detected in the virus preparation (Fig. 6, lane 1) or in extracts from uninoculated plants (Fig. 6, lane 2).

Laco and Beachy (1994) expressed the polymerase domain of RTBV P194 in insect cells using a baculovirus system and reported that it was processed into two products of 62 and 55 kDa with a common N-terminus. We also found a 55-kDa product and it is likely that our 65-kDa product corresponds to their 62-kDa protein.

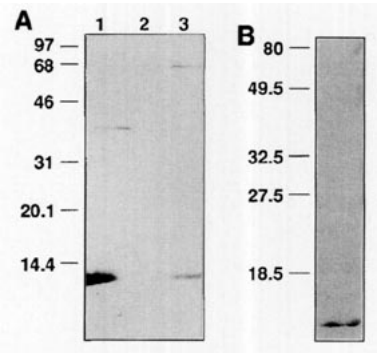

FIG. 5. Immobilized proteins probed with antiserum raised against the putative protease sequence of RTBV. (A) ECL Western blot. Lane 1, RTBV particles; lane 2, soluble proteins isolated from uninoculated rice plants; lane 3, soluble proteins isolated from RTBV-infected rice plants. (B) Chromogenic Western blot of RTBV particles. The ECL and prestained molecular weight markers are as described in the legend to Fig. 2.

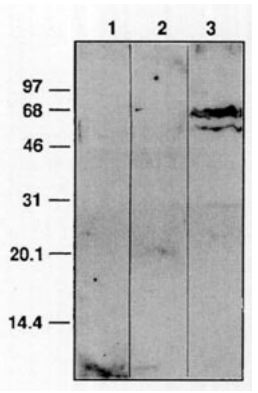

FIG. 6. ECL Western blot probed with antiserum raised against the putative reverse transcriptase sequence of RTBV. Lane 1, RTBV particles; lane 2, soluble proteins isolated from uninoculated rice plants; lane 3, soluble proteins isolated from RTBV-infected rice plants. The ECL molecular weight markers are described in the legend to Fig. 2.

Processing of P194

To determine which, if any, of the higher molecular weight proteins detected by Pprot, PRT and RTBV particle antisera were polyproteins encoding one or all three of these individual proteins, extracts from infected tissue were electrophoresed, blotted onto nitrocellulose, and probed in parallel with all three antisera. The resultant ECL blots showed that the 68-kDa polyprotein was common to the reverse transcriptase and protease (data not shown). This suggests that the RT/RNaseH 55-kDa product had not been cleaved from the N-terminal protease domain.

These Western blots also showed that a 37-kDa band reacted with both the particle and protease antisera (data not shown) which raises the possibility that this species comprises part coat protein and part protease. If such a product was incorporated into the particle it could explain the immunogold labeling of RTBV particles with protease antiserum. This would suggest that RTBV resembled retroviruses such as HIV1 in which the protease is involved in late stages of particle maturation and molecules of it are found in mature particles (see Luciw and Shacklett, 1993, for review). The larger species detected by the virus particle antiserum are likely to be due to lack of processing of regions N-terminal of the coat protein region.

P46

No proteins were immunodetected in virus particles or in extracts from infected or noninoculated rice plants using the antiserum raised against the product of ORF IV (P46) (data not presented). It is likely that P46 is expressed from a spliced mRNA (Fütterer *et al.*, 1994) and thus it is possible that this product is produced either in a low amount or at a specific stage of infection. Preliminary experiments using samples taken at different times after infection failed to reveal any product.

ACKNOWLEDGMENTS

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