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SEROLOGICAL RELATIONSHIPS BETWEEN RICE HOJA BLANCA VIRUS AND *Echinochloa* HOJA BLANCA VIRUS

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ABSTRACT

With the objective of determining whether rice hoja blanca virus (RHBV) and *Echinochloa* hoja blanca virus (EHBV) are serologically related, antisera were produced against the ribonucleoproteins of both viruses and used in several comparative studies. It was demonstrated that both viruses possess characteristics that separate them at this level. When compared in high resolution SDS-PAGE gels, it was observed that the EHBV nucleoprotein had a lower molecular mass than that of RHBV. The antiserum against RHBV recognizes the EHBV nucleoprotein in Western blot, indicating the presence of common epitopes. Reciprocal double immunodiffusion (OUCHTERLONY) tests showed the presence of both common and unique epitopes of the nucleoproteins of both viruses.

RESUMEN

Con el objetivo de determinar si el RHBV (Rice Hoja Blanca Virus) y el EHBV (*Echinochloa* Hoja Blanca Virus) se hallan relacionados serológicamente, se produjo un antisuero contra la proteína de nucleocápside del EHBV. Se demostró que ambos virus presentan características que los diferencian a este nivel. Al comparar la proteína de nucleocápside en geles SDS-PAGE de alta resolución, se observó que dicha proteína en el EHBV presenta una masa molecular menor que la del RHBV. El antisuero contra la proteína N del RHBV producido por ESPINOZA *et al.* (1992) reconoce la del EHBV por Western blot. Lo anterior indica que son similares y sugiere la presencia de epitopos comunes; además confirma que el EHBV presenta una menor masa molecular que el RHBV. Asimismo, el método de inmunodifusión doble de OUCHTERLONY mostró una identidad parcial de las nucleoproteínas de ambos virus, lo que confirma que ambos virus se encuentran serologicamente relacionados.

INTRODUCTION

Rice hoja blanca virus (RHBV) and *Echinochloa* hoja blanca virus (EHBV) belong to the tenuivirus group (FALK *et al.*1987). Both viruses are distributed in the majority of tropical and subtropical countries of America (GINGERY 1988, MORALES and NIESSEN 1985) occasionally reaching as far as Florida (ATKINS and ADAIR (1957), Mississippi (ATKINS *et al.* 1958) and Louisiana (ATKINS *et al.* 1960). *Echinochloa colona* (barnyard grass) is a highly common weed in rice production and has been described frequently as a host of EHBV (GÁLVEZ *et al.* 1962, FALK *et al.* 1987). MORALES and NIESSEN (1985) suggest that RHBV and EHBV, although related sero-

logically, are not identical and suggest that the virus present in *E. colona* could be a strain of RHBV. FALK *et al.* (1987) showed that these viruses could not be distinguished by Western blots or by proteolytic patterns of their nucleoprotein (N-protein) and major non-capsid proteins (NCP), indicating that RHBV and EHBV are very closely related.

However, comparative studies of the genomes of RHBV and EHBV indicated that these viruses present molecular characteristics that distinguish them (DE MIRANDA *et al.* 1996acde), particularly the size of the RNA-4, which is considerably smaller for EHBV than for RHBV.

The purpose of this investigation was to devise a serological method for identifying and distinguishing the viruses in their respective principal host.

MATERIAL AND METHODS

144 plants of *E. colona* and rice which presented typical symptoms of hoja blanca disease were collected at the experimental station Enrique Jimenéz Nuñez, the farm El Pelón de la Bajura (Cañas and Liberia respectively, Guanacaste, Costa Rica) and at various locations in Parrita (Puntarenas, Costa Rica). They were transported on ice and stored at -30 °C until further analysis.

Experimental controls were provided by healthy plants, grown from seed in the greenhouse. EHBV and RHBV ribonucleoprotein particles were purified using the minipreparation method of DE MIRANDA (1996a). Symptomatic leaves (± 0.5g) were crushed in a mechanical roller (Wenig & Koch, Germany) with 1 ml 0.1M Tris-HCl pH 7.5, 0.1% thioglycolic acid, 0.02 % sodium diethyldithiocarbamate (DIECA) and 0.1M NaCl. The extract was collected in 1.5 ml microfuge tubes, incubated on ice for one hour and centrifuged for 5 min at 10000 rpm. The supernatant was placed on 0.4 ml of 30% sucrose dissolved in the extraction buffer and the viral RNPs were collected in the lower phase by centrifuging for 4 hr at 14000 rpm at 4 °C.

The RNPs were denatured and analyzed in discontinuous 16 x 16 cm 12% SDS-PAGE gels (LAEMMLI 1970). The molecular mass of the N-proteins was estimated using a calibration curve established with molecular weight markers and the NCSA Gel reader computer program. The proteins were transferred to nitrocellulose paper (Western blot) according to SAMBROOK *et al.* (1989).

The antiserum against the EHBV RNP was produced in a single white New Zealand rabbit through intramuscular and subcutaneous injection of RNP purified successively by minipreparation from a single infected plant. One week after bleeding for pre-immune serum as control, the rabbit was injected with 0.5 mg of antigen emulsified in FREUND´S complete adjuvant, followed every two weeks by booster injections emulsified with FREUND'S incomplete adjuvant. After 6 boosters, the rabbit was bled from the marginal vein of the ear. The resulting antiserum was preadsorbed for 24 hours at 37 °C with lyophilized healthy plant extract, in order to eliminate possible antibodies produced against traces of plant protein contaminants.

The Western blots were incubated 16 hr at room temperature with a 1/1000 dilution of primary antibody in phosphate buffered saline phosphate (PBS) containing 0.01% Tween-20 and 5% fat-free powdered milk. The membrane was washed three times for 15 minutes with PBS and incubated 2 hours at room temperature with a 1/5000 dilution of alkaline phosphatase linked anti-rabbit-IgG monoclonal antibody in PBS-tween-milk. The membranes were washed with PBS and developed with 5% nitroblue tetrazolium (NBT) and 5% 5-Bromo-4-Chloro-3-Indolyl Phosphate (BCIP) according to SAMBROOK *et al.* (1989).

The OUCHTERLONY double immunodiffusion test was performed in Petri dishes with 1% agarose gels or 1.5% Phytagel®, both dissolved in 0.05M Tris-HCl (pH 7.2); 0.15M NaCl and 8 mM sodium azide. The gels were perforated with 5mm holes in a hexagonal arrangement with a single central hole. Antiserum against either EHBV or RHBV was placed in the central hole and 50 ml extracts of healthy plants and plants infected with EHBV or RHBV were placed in the peripheral holes.

RESULTS

The N-proteins of EHBV extracted from individual plants of *E. colona*, collected in three geographical different areas, showed characteristic patterns of migration different from RHBV, extracted from individual rice plants from the same locations (figure 1). This difference is not evident in minigels (figure 2), which may explain why FALK *et al.* (1987) did not find differences in the mobilities of the N-proteins of MStV, RHBV and EHBV.

The approximate molecular mass of the N-protein of RHBV and EHBV was determined from their migration in five different 16 x 16 cm SDS-PAGE gels. The RHBV N-protein has a calculated mass of 35689 ± 655 Da, while the EHBV N-protein is 34333 ± 706 Da. The theoretical mass for this protein, calculated from RNA sequence data, was determined to be 35257 Da for RHBV and 34927 Da for EHBV. The difference between the experimental and theoretical molecular mass was not significant for either protein, indicating that there is little, if any, post-translational protein modification for the N-proteins.

Using this difference as a marker for the presence of either (or both) EHBV and RHBV it was determined that the EHBV N-protein band was only found in the *E. colona* plants extracts, and the RHBV N-protein band only in rice plant extracts. The Western blot (figure 3) confirms that the protein bands seen in figure 2 correspond to the N-proteins of EHBV and RHBV, and that the antiserum to RHBV RNPs recognizes the EHBV N-protein, indicating the presence of common antigenic epitopes between EHBV and RHBV. However, the reaction with the EHBV N-protein is less strong that with the RHBV N-protein, even though similar protein quantities were loaded in each lane, suggesting that the cross-reaction is not total.

This was confirmed by the results from the double immunodiffusion tests (figure 4). The antibodies against the common epitopes in both viral

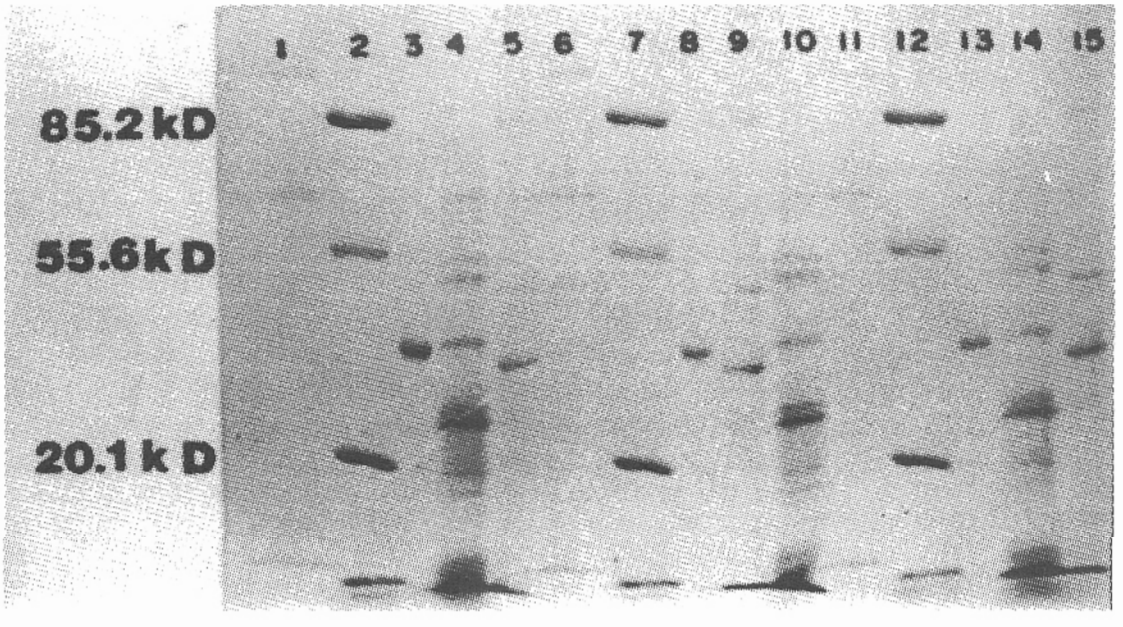

Figure 1. Patterns of migration from RNPs of the RHBV and EHBV, partially purified by the method of minipreparation in a gel SDS-PAGE 12% 16 x 16 cm. Tracks 2, 7 and 12: weight molecular markers; tracks 1, 6 and 11: healthy plant; tracks 4, 10, 14 and 5, 9 and 15: rice and *Echinochloa* infected respectively. RNPs of highly purified RHVB were used as controls (tracks 3, 8 and 13)

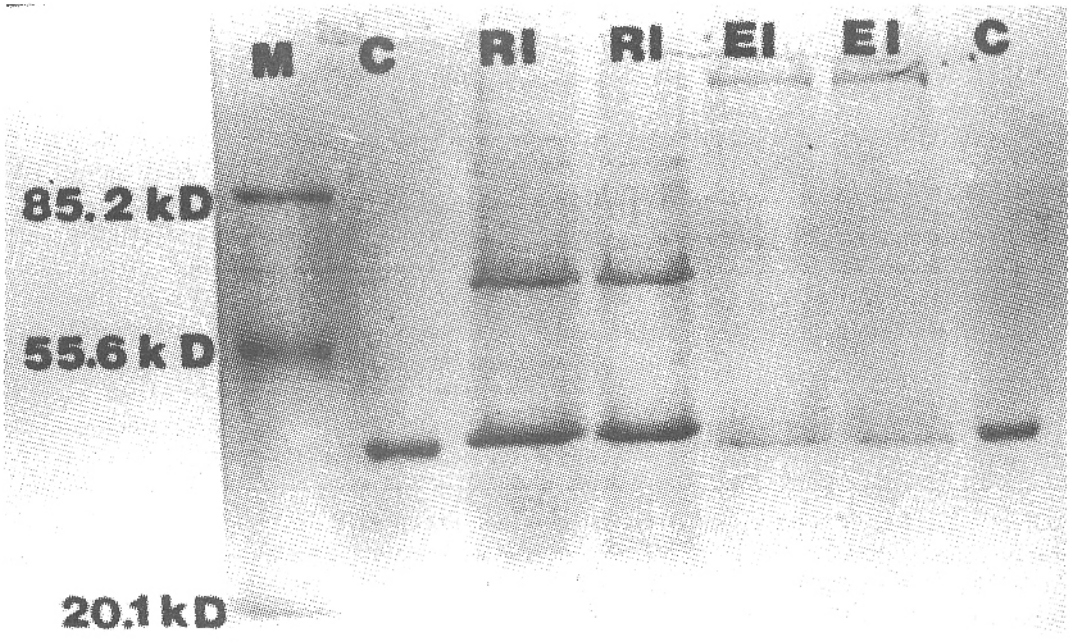

Figure 2. Patterns of migration from RNPs of the RHBV and EHBV, partially purified by the method of minipreparation in a gel SDS-PAGE 12% 7 x 5 cm. Tracks M: weight molecular marker; C: RHVB highly purified; RI and EI: rice and *Echinochloa* infected respectively.

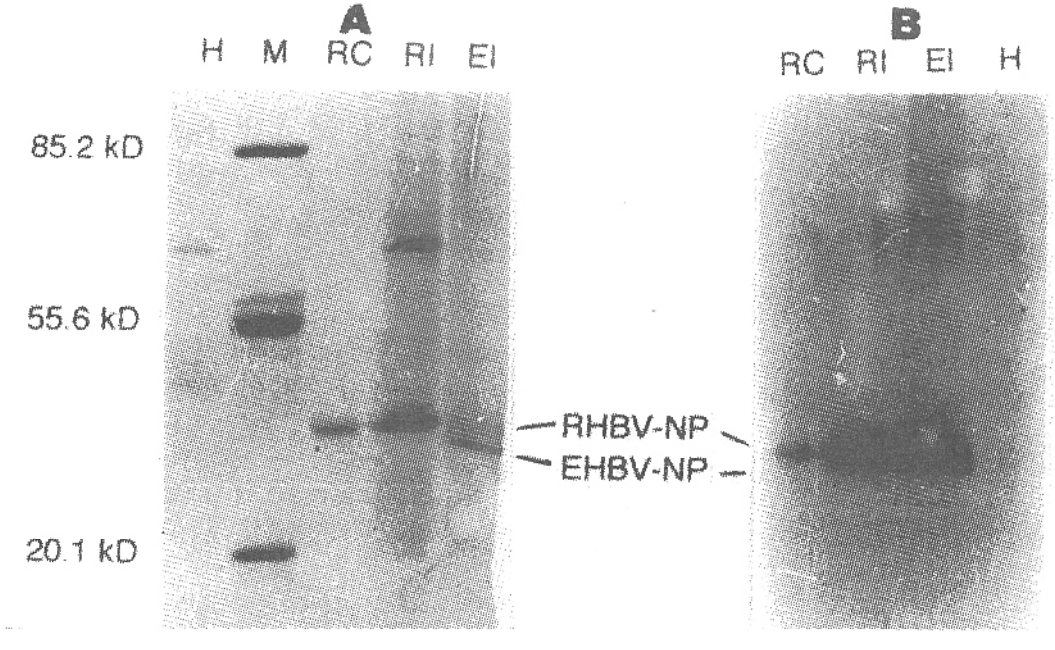

Figure 3. A) Patterns of migration from RNPs of the RHBV and EHBV, partially purified by the method of minipreparation in a gel SDS-PAGE 12% 16 x 16 cm. B) Western Blot of a similar gel, probed with antiserum raised against the RHBV nucleoprotein. H= Healthy plants; M= weight molecular marker; RC= RNPs of RHVB highly purified as controls; RI and EI = rice and *Echinochloa* infected respectively.

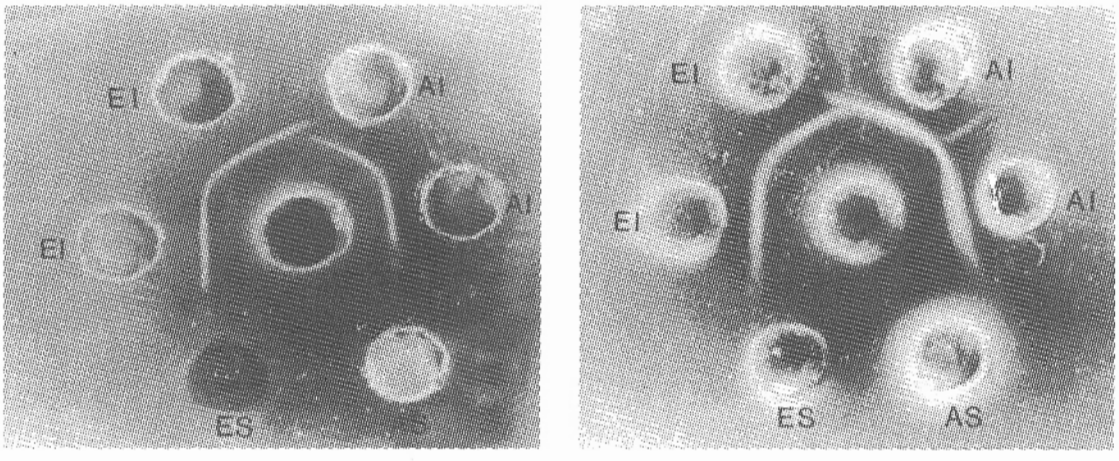

Figure 4. Double immunodiffusion "Ouchterlony" tests of the antisera raised against RHBV (a) and EHBV (b) ribonucleoprotein. In each case, the antiserum is placed in the center and the plant extracts are placed peripherally. AI and EI refer to rice and *E. colona* infected, while AS and ES refer to rice and *E. colona* healthy.

proteins formed precipitation line when the antiserum against one virus diffuses into the RNPs of the other virus. Antibodies against unique epitopes continue their diffusion until encountering the appropriate antigens from the homologous virus, forming a characteristic spur between the heterologous and homologous virus extracts. This spur is absent between adjacent homologous (or heterologous) extracts. Neither antiserum formed any precipitation with the extracts from healthy plants. The majority of authors consulted (MORALES and NIESSEN 1983, RIVERA and RAMÍREZ 1991, HIBINO 1986) utilized agar or agarose as the diffusion gel. However, we found that the Phytagel yields a more transparent gel, with better resolution of the precipitin bands. The only disadvantage was that, with the gel concentration used, Phytagel required more time for the bands to develop.

DISCUSSION

The serological similarity between EHBV and RHBV contradicts the results of MORALES and NIESSEN (1983), who did not find any serological reaction between antiserum against RHBV RNPs and extracts of *E. colona* with viral symptoms. This suggests that their experiments may have involved a different (tenui) virus of *E. colona* (L. CALVERT, pers. comm.). FALK *et al.* (1987) did find serological similarity between EHBV and RHBV, but did not investigate their possible differences. The serological evidence presented here complements the nucleic acid data of RHBV and EHBV (DE MIRANDA 1996 a,b,c,d,e), which suggest that although EHBV and RHBV have a close evolutionary relationship, they are distinct viruses, transmitted by different delphacid vectors to a different range of host plants (MADRIZ *et al.* 1998).

The practical value of these conclusions is that the virus found in *E. colona* is distinct from RHBV, that infected *E. colona* plants present no immediate danger to rice production (unlike RHBV which has enormous economic importance), and that there is no need for the chemical elimination of *E. colona* for reasons related to virus infection.

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