Development of Serological Procedures for Rapid, Sensitive, and Reliable Detection of Rice Grassy Stunt Virus

H. T. HSU, Microbiologist, USDA, Agricultural Research Service, Beltsville Agricultural Research Center, Beltsville, MD 20705; and H. HIBINO, Plant Virologist, and P. Q. CABAUATAN, Senior Research Assistant, International Rice Research Institute, Manila, Philippines

ABSTRACT

Hsu, H. T., Hibino, H., and Cabauatan, P. Q. 1990. Development of serological procedures for rapid, sensitive, and reliable detection of rice grassy stunt virus. Plant Dis. 74:695-698.

Hybridoma cell lines secreting IgG2a antibodies reactive to two strains of rice grassy stunt virus (RGSV) were established by the limiting dilution cloning technique. Serological detection of RGSV with mouse monoclonal antibodies and rabbit polyclonal antibodies was compared by enzyme-linked immunosorbent assay (ELISA), dot-blot immunoassay (DBI), and a latex agglutination test (LAT). A dilution endpoint of approximately 10^{-3} was obtained for both mouse monoclonal antibodies and rabbit polyclonal antiserum by LAT. The RGSV antigen in 2.5 μl of a 10^{-3} dilution of infected sap could be detected by DBI when alkaline phosphatase-labelled antibodies were used. Using double antibody sandwich ELISA, an approximately 10-fold increase in sensitivity for RGSV detection was achieved. For practical purposes, however, the LAT provides the simplest procedure for routine detection of RGSV.

Rice (*Oryza sativa* L.) is one of the major food crops in many parts of the world. Production of rice is seriously affected by virus infection (14). Among the virus diseases, rice grassy stunt occurs widely in rice-growing regions in the South, Southeast, and East Asia. It is characterized by stunting of plants and proliferation of short, erect, pale green leaves.

Rice grassy stunt virus (RGSV; 4) is transmitted in a persistent manner by the rice brown planthopper (*Nilaparvata lugens* Stål). During 1982 and 1983 in the Philippines, the occurrence of a new strain (RGSV-2) in rice cultivars carrying the resistance gene G_3 to the ordinary strain (RGSV-1) was noticed (3,10). Leaf yellowing symptoms caused by RGSV-2 in rice are similar to those of tungro-infected rice plants (3).

Present address of second author: Plant Pathologist, National Agriculture Research Center, Tsukuba, Ibaraki 305, Japan.

Cooperative investigation of the USDA-ARS, Beltsville Agricultural Research Center, and the International Rice Research Institute, Los Banos. Supported in part by the U. S. Agency for International Development, Program in Science and Technology Cooperation Grant 492-5542-G-55-4097.

Accepted for publication 6 April 1990 (submitted for electronic processing).

Rice grassy stunt virus is often not accurately diagnosed. The lack of correct identification of rice viruses continues to be a major obstacle in the improvement of rice production. Recognition of symptoms on affected rice plants is the primary method of diagnosis of many rice virus diseases (12). Results, however, are often inconclusive. Although many rice viruses are transmitted by specific insect vectors, routine tests for the detection of a particular virus are labor-intensive and time-consuming. These transmission tests can be performed only when the appropriate vector colonies are available.

The availability of serological reagents for diagnosis of rice grassy stunt is limited. We report the generation of mouse hybridoma cell lines, production of monoclonal antibodies, and use of the antibodies for detection and identification of RGSV, both in infected plants and in viruliferous vector planthoppers. Preliminary reports have been published (1,7).

MATERIALS AND METHODS

Virus and rabbit polyclonal antisera. RGSV-1 and RGSV-2 were isolated from individual infected rice plants collected in the Philippines (3,4). Using vector planthoppers, *N. lugens*, the RGSV cultures were established and maintained in the rice cultivar Taichung Native 1 at the International Rice Research Institute (IRRI).

Purified RGSV-1 and RGSV-2 viral antigens were prepared at IRRI (4) and were sent from the Philippines, with the permission of APHIS, to the United States for production of mouse monoclonal antibodies (McAbs). Rabbit polyclonal antisera for RGSV-1 and RGSV-2 were those prepared previously (3,4).

Extraction of RGSV antigens from plants and vectors. One part rice leaves infected with either RGSV-1 or RGSV-2 were homogenized in nine parts buffer (0.02 M sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl and 0.05% Tween 20) for enzyme immunosorbent assays or dot-blot immunoassays and in 0.05 M Tris-HCl buffer, pH 7.2, for latex tests. Noninfected leaves served as controls. To prepare extracts from vectors, planthoppers were kept on RGSV-2-infected plants for a 4-day acquisition access feeding (3). Individual insects were homogenized with 0.25 ml of a 0.02 M sodium phosphate buffer containing 2% polyvinylpyrrolidone 10,000 (PVP 10,000). Nonviruliferous planthoppers that did not have access to a diseased plant served as controls.

Cell culture, hybridoma formation, and production of monoclonal antibodies. Materials and methods used in preparation of the immune spleen cells, hybridization with myeloma cells, screening, selection, cloning, and stabilization of desirable McAb-secreting hybridomas and determination of immunoglobulin isotypes were the same as those described for barley yellow dwarf virus (6). Mice were individually immunized with either RGSV-1 or RGSV-2.

Antibody purification. RGSV mouse monoclonal antibodies (IgG2a) were purified by affinity chromatography through a Protein A-sepharose column as previously described (5). Rabbit polyclonal immunoglobulins were purified according to a procedure reported previously (2).

Enzyme-labelled immunosorbent assays (ELISA). Purified RGSV-specific mouse immunoglobulins or rabbit immunoglobulins were conjugated to alkaline phosphatase according to the method previously described (2). Conjugates were diluted in a phosphate-buffered saline solution (PBS, 0.01 M phosphate, 0.15 M NaCl, pH 7.3) and were adjusted to an antibody concentration of 0.5 μg/ml. Enzyme-labelled goat anti-mouse immunoglobulins and enzyme-labelled goat anti-rabbit immunoglobulins were purchased from Kirkegaard and Perry Laboratories, Inc. (Gaithersburg, MD) or Sigma Chemical (St. Louis, MO).

Monoclonal antibodies produced in this study were selected for detection of RGSV in double antibody sandwich ELISA and dot-blot immunoassays with a procedure similar to the one used in selecting barley yellow dwarf virus monoclonal antibodies (6). Polystyrene or polyvinyl chloride plates were coated with rabbit anti-RGSV immunoglobulins at 1 μg/ml in 0.05 M carbonate buffer at pH 9.6. The plates were blocked with 1% bovine serum albumin in PBS for 30 min before incubation with 0.1 μg/ml RGSV antigens and were then used for hybridoma screenings either using alkaline phosphatase (AP)-labelled goat anti-mouse immunoglobulins or horseradish peroxidase (HRP)-labelled goat anti-mouse immunoglobulins as previously described (6).

RGSV antigens from infected rice plants or viruliferous vector planthoppers were detected with the double antibody sandwich ELISA. Polystyrene plates were coated with purified RGSV-specific mouse monoclonal antibodies or rabbit antibodies, 1 μg/ml in 0.05 M carbonate buffer at pH 9.6, followed by a blocking step (8). Plates were incubated with antigen extracts and then with RGSV-specific AP-labelled mouse immunoglobulins (for mouse antibody coated plates) or AP-labelled rabbit immunoglobulins (for rabbit antibody coated plates).

For AP-antibody conjugates, *p*-nitrophenyl phosphate at 1 mg/ml in substrate buffer (97 ml of diethanolamine, 0.1 g of $MgCl_2 \cdot 6H_2O$, 900 ml of distilled water, pH 9.8) was used. Immediately before use, 50 μl of 3% H_2O_2 was added to 50 ml of distilled water containing 5 mg of *o*-phenylenediamine as substrate for HRP-antibody conjugates.

Dot-blot immunoassays (DBI). A nitrocellulose (NC) membrane (Schleicher & Schuell, Inc., Keene, NH), 0.45-m pore size, previously immersed in Tris-buffered saline solution (TBS, 0.05 M Tris-HCl, 0.15 M NaCl, pH 7.5) was secured in either a Bio-Dot microfiltration apparatus (Bio-Rad Laboratories, Richmond, CA) or a minifold apparatus (Schleicher & Schuell, Inc., Keene, NH). A small amount (10 μl) of leaf extract was applied to each well under vacuum. The NC membranes were then immersed in a blocking solution (TBS containing 0.05% Tween 20, 2% PVP 10,000 and 2% bovine serum albumin) for 30 min before a 60-min incubation in rabbit polyclonal antiserum or mouse monoclonal antibodies at 1/500 dilution in a blocking solution. Following a rinse in distilled water and two washings, 10 min each in blocking solution, the blots were incubated for 60 min in enzyme-labelled second antibodies at 1/2,000 dilutions in a conjugate buffer (0.05 M Tris-HCl, 0.1 M NaCl, 0.5 mM $MgCl_2$, pH 7.5). The blots were then rinsed once with distilled water and washed twice, 10 min each in conjugate buffer, before immersion in an appropriate substrate solution.

For AP-labelled antibodies, the NC membranes were incubated in a freshly prepared color development solution containing 0.33 mg/ml of nitro blue tetrazolium and 0.17 mg/ml of 5-bromo-4-chloro-3-indolyl phosphate-*p*-toluidine salt. For HRP-labelled antibody conjugates, the blots were incubated in TBS containing 0.5 mg/ml of 4-chloro-1-naphthol, 0.05% hydrogen peroxide. After color development, the reaction was stopped by washing the NC membrane in 0.01 M Tris-HCl containing 0.05 M EDTA, pH 7.5.

Latex agglutination test (LAT). The latex test method was previously described (13). Latex particles (Difco Bacto-Latex, 0.81 μm size) were sensitized with either rabbit anti-RGSV immunoglobulin or ascitic fluid of mouse monoclonal antibodies at a 1/1,000 dilution. Approximately 25 μl of leaf extract and 25 μl of sensitized latex suspension were placed in a well of an ELISA plate, and the plate was agitated on a shaker for 1 hr. The presence of the virus antigens was indicated by the clumping of latex particles when examined under a light microscope.

RESULTS

Hybridomas and antibodies. Two fusions, one each for RGSV-1 and RGSV-2, were made. Initial selections of RGSV monoclonal antibodies were based on ELISA results using either AP-labelled or HRP-labelled goat anti-mouse immunoglobulins. A total of 35 hybridoma cultures that were positive for RGSV but were negative to the extract of healthy rice leaves were obtained from 1,749 cultures of hybrids screened. Eleven were from the fusion using RGSV-1 immunized splenocytes, and 24 were from RGSV-2 immunized splenocytes.

Although attempts were made to produce monoclonal antibodies for differentiation of the two strains of RGSV, clonings of all RGSV-positive hybridomas resulted in seven stable cell lines all originating from the same culture derived from the RGSV-1 fusion. The seven cell lines all secrete IgG2a immunoglobulins, which recognize both RGSV-1 and RGSV-2.

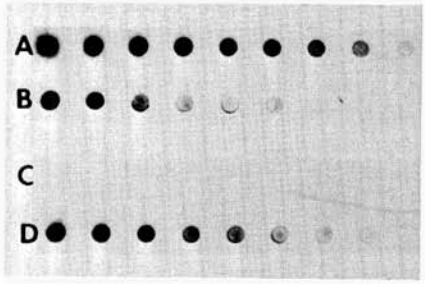

Fig. 1. Determination of dilution endpoint titer of viral antigens in healthy leaf tissue **(A and C)** and in rice grassy stunt virus (RGSV)-infected leaf tissue **(B and D)** with rabbit polyclonal **(A and B)** or mouse monoclonal antibodies **(C and D)**. A two-fold dilution of tissue extract starting 10^{-1} from left was applied to nitrocellulose membranes with a dot-blot apparatus under vacuum.

AP conjugate vs. HRP conjugate. AP-labelled goat anti-mouse immunoglobulins were compared with HRP-labelled goat anti-mouse immunoglobulins in both ELISA and DBI for assays of RGSV-2 in infected rice leaves. ELISA titration of RGSV showed that the dilution endpoints of viral antigens in infected leaves were about 10^{-4} when either AP- or HRP-labelled goat anti-mouse immunoglobulins were employed (*data not shown*). Healthy controls at the 10^{-1} dilution, the lowest dilution tested, were negative for both enzyme conjugates. In DBI tests, a dilution endpoint of about $10^{-3.1}$ was obtained for RGSV infected leaves, and negative results were obtained for healthy controls when AP conjugates were employed. Using HRP conjugates in a DBI test, strong positive reactions were obtained for both leaf tissue extracts of RGSV-infected rice plants and healthy controls (*data not shown*). A similar strong positive reaction occurred with the RGSV-infected tissue extract and with control healthy tissue extract blots on NC membranes that were not incubated with primary antibodies or HRP-immunoglobulin conjugates (*data not shown*).

Rabbit polyclonal antiserum vs. mouse monoclonal antibody. Using AP-immunoglobulin conjugates, the feasibility of use of mouse monoclonal antibodies for detection of RGSV infection was compared with rabbit polyclonal antiserum in DBI tests. When rabbit polyclonal antiserum was used in DBI, strong positive reactions were

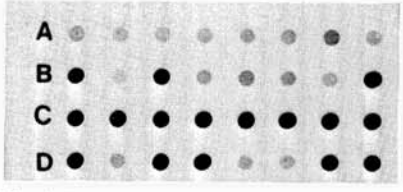

Fig. 2. Dot-blot immunoassays of rice grassy stunt virus antigens in extracts of infected leaf tissues. Ten μl of tissue extracts at 10^{-1} dilution were applied to a nitrocellulose membrane with a dot-blot apparatus under vacuum. Subsequent steps including blocking, washing, and reacting with primary antibodies (mouse monoclonal antibodies) and second antibodies (alkaline phosphatase-labelled goat anti-mouse immunoglobulin) were carried out while the membrane was still secured inside the dot-blot apparatus with vacuum attached to it. The blot was removed after the last washing and incubated in a substrate solution. Healthy controls **(A)** developed faint color and infected extracts **(C)** developed strong color.

observed for extracts from RGSV-infected leaf tissues and from healthy control plant extracts, thus making results difficult to interpret (Fig. 1). DBI tests using mouse monoclonal antibodies, on the other hand, resulted in positive reactions for RGSV-infected tissue extracts and negative reactions for healthy controls (Fig. 1). The virus-infected tissue had an RGSV dilution endpoint of $10^{-3.1}$.

Attempts were made to determine if a complete DBI test procedure including sample application, blocking, washing, and reagent incubation could all be accomplished under vacuum inside a dot-blot manifold before substrate incubation while membranes were still in place. The NC membrane was then removed from the apparatus before being incubated in a substrate solution. A strong positive response was noted on dots spotted with RGSV-infected leaf extracts. Faint color of positive reaction on healthy control samples was also noticed (Fig. 2). The experiment was accomplished within 30 min from the application of samples. In another experiment, NC membranes were removed from the dot-blot apparatus after sample application and subsequent procedures of blocking, washings, and addition of reagents were carried out in a petri dish. Color developed only on dots spotted with RGSV-infected leaf extracts. The intensity of color was not as strong as those of NC membranes processed while still attached to the apparatus. Healthy control dots did not develop color (Fig. 3). The entire test, however, took 4 hr to complete.

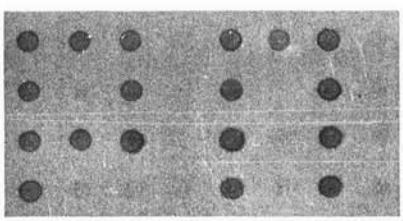

Fig. 3. Dot blot immunoassays of rice grassy stunt virus antigens in extracts of infected leaf tissue. Ten μl of tissue extracts at 10^{-1} dilutions were applied to a nitrocellulose membrane with a dot-blot apparatus under vacuum. The membrane was removed from the apparatus. Subsequent steps were carried out in a petri dish. Color developed on spots that were dotted with extracts from infected leaf tissues, whereas those spotted with extracts from healthy leaf tissues remained colorless.

Comparison of ELISA, DBI, and LAT. Using optimal conditions for each test, the sensitivities of ELISA, DBI, and LAT for detection of RGSV infection of rice plants were compared (Table 1). RGSV dilution endpoints of the infected leaf extract by double antibody sandwich ELISA were $10^{-4.3}$ and 10^{-4} with rabbit polyclonal antibodies and mouse monoclonal antibodies, respectively. Dilution endpoints of the RGSV-infected leaf extract by LAT were about 10^{-3} with either rabbit polyclonal or mouse monoclonal antibodies. When mouse monoclonal antibodies were used, the size of latex particle clumps was smaller than those obtained with rabbit polyclonal antibodies. It also took longer to form clumps. For DBI, an RGSV dilution endpoint of $10^{-3.1}$ was obtained for the infected leaf extract with mouse monoclonal antibodies (Table 1 and Fig. 1). Although a $10^{-3.4}$ DBI dilution endpoint was obtained with rabbit polyclonal antiserum, the healthy control plant extract also developed strong color (Table 1 and Fig. 1).

Virus detection in *N. lugens* planthoppers. RGSV was detected in 15 out of 360 vectors that had an acquisition access feeding on RGSV-2-infected plants. ELISA A_{405} values using monoclonal antobodies ranged from 0.08 to 0.43 compared to 0.01 or lower for the controls.

DISCUSSION

Seven cloned hybridoma cell lines were all selected from a culture that originated from the same well in a 96-well culture plate. Isotype by double agar gel diffusion tests showed that all seven hybridoma cell lines secrete IgG2a immunoglobulins. Double antibody sandwich ELISA tests showed that the monoclonal antibodies produced by each cell line all recognize RGSV-1 and RGSV-2. It is likely that these are the same cell lines and that they secrete the same antibodies. A total of 1,246 wells were seeded in a fusion from which 774 hybrid cultures were obtained (*data not shown*). Only 11 cultures secreted RGSV specific antibodies. The chance that two or more fused hybrids occupied a single well in this experiment cannot be ruled out.

ELISA was about 10 times more sensitive than DBI or LAT for detection of RGSV infection. However, ELISA was not as economical as the other two methods. Employing mouse monoclonal antibodies, a dilution endpoint of about 10^{-3} was achieved by both DBI and LAT for detection of RGSV infection. Unlike rabbit polyclonal antiserum in DBI, which developed false positive reactions with healthy controls, test results with mouse monoclonal antibodies in DBI are reliable.

Proper selection of enzyme systems is important in DBI. Our results show that use of a peroxidase system in DBI is not suitable for assays of viruses in extracts of rice leaves. This is because of the presence of peroxidase(s) in the extracts of rice leaf tissue. Alkaline phosphatase, on the other hand, provides an excellent substitute for horseradish peroxidase in DBI in detecting viruses from rice plants. For assays of cytoplasmic polyhedrosis virus from gypsy moth, horseradish peroxidase, however, is a suitable enzyme in DBI, because extracts of healthy insect larvae contain alkaline phosphatase (H. T. Hsu, *unpublished data*).

Although economical, LATs utilizing sensitized inert particles are not commonly utilized in plant virus identification (9,11,13). LAT procedures are very practical in that a batch of a sensitized latex preparation can be used in numerous tests for a long period. The reaction is rapid, usually within 30 to 60 min from the beginning of a test. The method is sensitive, and the results are reliable.

In recent trials using the mouse monoclonal antibodies, ELISA detected several Philippines RGSV isolates that induced different symptoms in rice (P. Q. Cabauatan, *unpublished data*) and RGSV isolates, one each in Indonesia and Thailand (H. Hibino, *unpublished data*).

The monoclonal antibodies to RGSV can be utilized in the study of RGSV epidemiology. These monoclonal antibodies are currently being used in general disease surveys and monitoring viruliferous planthoppers caught in light traps in the Philippines.

Table 1. Detection of rice grassy stunt virus in infected plant tissue by enzyme-linked immunosorbent assay (ELISA), latex agglutination test (LAT), and dot-blot immunoassay (DBI) with mouse monoclonal (MA) and rabbit polyclonal (PA) antibodies

Serological assay	IgG dilution[a] (1st antibody)		Conjugate dilution (2nd antibody)		Sap dilution	
	PA	MA	PA	MA	PA	MA
ELISA	1,000	1,000	1,000	1,000	20,000	10,000
LAT	1,000	1,000	...	...	1,024	1,024
DBI	1,000	1,000	2,000[b]	2,000[c]	2,560[d]	1,280

[a] Reciprocal of dilution.
[b] Goat anti-rabbit IgG-alkaline phosphatase.
[c] Goat anti-mouse IgG-alkaline phosphatase.
[d] Strong nonspecific reaction.

LITERATURE CITED

1. Cabauatan, P. Q., Hibino, H., and Hsu, H. T., 1988. Dot-blot immunoassay (DBI) for detecting rice grassy stunt virus (RGSV). Int. Rice Res. Newsl. 13(4):34-35.
2. Clark, M. F., and Adams, A. N. 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the

detection of plant viruses. J. Gen. Virol. 34:475-483.
3. Hibino, H., Cabauatan, P. Q., Omura, T., and Tsuchizaki, T. 1985. Rice grassy stunt virus strain causing tungrolike symptoms in the Philippines. Plant Dis. 69:538-541.
4. Hibino, H., Usugi, T., Omura, T., Tsuchizaki, T., Shohara, K., and Iwasaki, M. 1985. Rice grassy stunt virus: A planthopper-borne circular filament. Phytopathology 75:894-899.
5. Hsu, H. T., Aebig, J., Civerolo, E. L., and Helkie, C. 1982. Enzyme conjugated protein A: A universal reagent for indirect enzyme-linked immunosorbent assays (ELISA). (Abstr.) Phytopathology 72:938.
6. Hsu, H. T., Aebig, J., and Rochow, W. F. 1984. Differences among monoclonal antibodies to barley yellow dwarf viruses. Phytopathology 74:600-605.
7. Hsu, H. T., Cabauatan, P. Q., and Hibino, H. 1986. Mouse monoclonal antibody to rice grassy stunt virus and its use for disease detection. (Abstr.) Phytopathology 76:1132.
8. Hsu, H. T., Franssen, J. M., van der Hulst, T. C., Derks, A. F. L. M., and Lawson, R. H. 1988. Factors affecting selection of epitope specificity of monoclonal antibodies to tulip breaking potyvirus. Phytopathology 78:1337-1340.
9. Khan, M. A., and Slack, S. A. 1978. Studies of the sensitivity of a latex agglutination test for the serological detection of potato virus S and potato virus X in Wisconsin. Am. Potato J. 55:627-637.
10. Khush, G. S., and Ling, K. C. 1974. Inheritance of resistance to grassy stunt virus and its vector in rice. J. Hered. 65:134-136.
11. Koenig, R., and Bode, O. 1978. Sensitive detection of Andean potato latent and Andean potato mottle viruses in potato tubers with the serological latex test. Phytopathol. Z. 92:275-280.
12. Ling, K. C. 1972. Rice Virus Diseases. International Rice Research Institute, Los Banos, The Philippines. 142 pp.
13. Omura, T., Hibino, H., Usugi, T., Inoue, H., Morinaka, T., Tsurumachi, S., Ong, C. A., Putta, M., Tsuchizaki, T., and Saito, Y. 1984. Detection of rice viruses in plants and individual insect vectors by latex flocculation test. Plant Dis. 68:374-378.
14. Ou, S. H. 1985. Rice Diseases. 2nd ed. Commonwealth Agricultural Bureaux, Kew, Surrey, England. 380 pp.