Identification, Purification, and Serological Detection of the Major Noncapsid Protein of Rice Grassy Stunt Virus

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ABSTRACT

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The major noncapsid protein (NCP) of rice grassy stunt virus (RGSV) was detected by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and purified by differential pH precipitation and a single ultracentrifugation step. Purified NCP formed needle-shaped crystals at pH 5.0 to 6.0. The NCP is composed of a single protein with a molecular mass of 24 kDa. The yield of NCP was about 20 mg per 100 g of tissue. Because antisera prepared against purified NCP reacted with purified RGSV ribonucleoprotein (RNP) particles, NCP was further purified by SDS-PAGE. The NCP antisera thus obtained reacted strongly and specifically with purified NCP and infected plants in indirect enzyme-linked immunosorbent assay (ELISA). Purified RGSV RNPs and sap from healthy rice (*Oryza sativa* L.) plants did not elicit a reaction. The RGSV NCP antisera also reacted with the NCP of rice stripe virus. NCP was not detected in insects by these methods, although RGSV was detected in planthoppers by double-antibody sandwich ELISA. Western blot analysis also could detect NCP in extracts of infected plants but not in insects. These results are consistent with those reported for maize stripe virus. RGSV NCP is very similar to the NCP produced by other tenuiviruses.

Rice grassy stunt virus (RGSV) is transmitted by the planthopper *Nilaparvata lugens* (Stal.). Rice grassy stunt is one of the most destructive viral diseases of rice (*Oryza sativa* L.) in rice-growing regions in southern, Southeast, and eastern Asia (10,11,21).

RGSV is a member of the genus *Tenuivirus* (20); other members are rice stripe virus (RSV), maize stripe virus (MSpV), and rice hoja blanca virus (RHBV). *Echinochloa* hoja blanca virus (EHBV) and winter wheat mosaic virus (WWMV) are tentative members (20). Tenuiviruses have fine-stranded viral particles, referred to here as ribonucleoproteins (RNPs), and produce large amounts of noncapsid protein (NCP) in leaves of infected plants (7,8,23). Ultrathin sections of tissues from tenuivirus-infected plants showed crystalline aggregates of different sizes and shapes (8,22,25). These inclusion bodies are composed of proteins but are not associated with RNPs; they have been referred to as the major NCP.

The production of NCP has been reported for RSV (14,15,17), MSpV (5,6,9,12), RHBV, EHBV (4,23,24), and WWMV (1). It was also reported that the NCP produced by these viruses can be used for serological detection, and it has been used for assaying virus-infected plants (4,5). RSV NCP is readily detectable in both infected plants (15,17) and viruliferous insects (26). The MSpV NCP has been detected in infected plants but not in viruliferous planthoppers (6). Although the presence of inclusion bodies in RGSV-infected plants has been reported (22,25), RGSV has not been previously reported to produce NCP.

We report the detection of NCP in RGSV-infected plants, its purification, and a serological assay for indexing RGSV-infected plants using antiserum produced to the purified major NCP.

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MATERIALS AND METHODS

Maintenance of vector and virus. Virus-free *N. lugens* that had been reared for several generations on healthy rice plants (cultivar TN1) were obtained from the virology greenhouse at IRRI, Philippines. The RGSV isolate, designated RGSV-SC, was collected in a field in South Cotabato, Philippines (19). To propagate the isolate, virus-free nymphs were allowed to feed on diseased plants for a minimum of 4 days. Planthoppers were transferred to healthy 7- to 10-day-old TN1 seedlings for 6 days for virus incubation in the vectors. Viruliferous insects (three per seedling) were then kept on new TN1 healthy seedlings for 24 h. Inoculated seedlings were individually transplanted into clay pots and were observed for symptoms in the greenhouse. Plants were harvested about 4 weeks after inoculation. Virus particles were purified and RGSV antisera were produced as described previously (11).

Detection of the RGSV NCP. Rice leaf blades (1 g) were homogenized in 4 ml of 0.02 M phosphate buffer (PB), pH 7.5. The homogenate was centrifuged at 17,500 *g* for 10 min. The supernatant was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) by Laemmli's method (18). The samples were dissociated in 0.25 M tris-HCl buffer (pH 6.8) containing 2% SDS and 5% 2-mercaptoethanol by heating at 100°C for 2 to 3 min. After electrophoresis, the gel was stained with Coomassie brilliant blue G250.

Viruliferous planthoppers were collected and frozen at –20°C for 2 h. Each sample was homogenized with 300 µl of PBST (0.02 M phosphate, 0.15 M NaCl, 0.05% Tween 20) with 2% polyvinylpyrrolidone (PVP) 40. Extracts were divided into three aliquots. The first two aliquots were tested for the presence of RGSV RNP and NCP by double-antibody sandwich (DAS) and indirect enzyme-linked immunosorbent assay (ELISA), respectively; the remaining aliquot was kept in the freezer for Western

blot analysis. Individual and pooled (two, three, four, five, and 10 insects per well) viruliferous planthoppers were tested. The same method was followed for virus-free planthoppers.

Extracts of individual and pooled viruliferous planthoppers that showed positive reaction for RGSV by ELISA were processed for SDS-PAGE and further subjected to Western blotting. Western blot analysis was done by Burnette's method (2) with 12% SDS-PAGE gels. Proteins were transferred onto 0.45-μm nitrocellulose membranes with a Trans-Blot cell apparatus (Bio-Rad Laboratories, Richmond, CA) according to the manufacturer's instructions. The membranes were first incubated with virus-specific rabbit immunoglobulin (IgG) and then with alkaline-phosphatase-conjugated goat anti-rabbit IgG. The RGSV NCP bands were visualized by the addition of nitro blue tetrazolium and bromo-chloro-indolyl phosphate.

Purification of RGSV NCP. RGSV NCP was concentrated by the differential pH precipitation procedure described by Gingery et al. (8,9), with slight modification. About 100 g of fresh tissue of RGSV-infected plants was homogenized in a phosphate-citrate buffer (PCB, a mixture of 0.2 M KH_2PO_4 and 0.1 M citric acid), pH 5.0 (3 ml of buffer per 1 g of tissue), in a leaf and bud press (Erich Pollahne, Wennigsen, Germany). The extract was centrifuged at 11,000 *g* for 10 min. Pellets were dissolved in PCB, pH 7.0 (1 ml per gram of tissue), and centrifuged at 11,000 *g* for 30 min. An equal volume of PCB, pH 3.0, was then added to lower the pH of the supernatant. The mixture was incubated overnight at 4°C and then centrifuged at 11,000 *g* for 10 min. Solubilization and precipitation steps were repeated. The final pellets were dissolved in PCB at pH 7.0, and the suspension was ultracentrifuged at 68,000 *g* for 1.5 h. The supernatant was then dialyzed against distilled water and lyophilized.

NCP obtained from differential pH precipitation was further purified by SDS-PAGE. Samples were suspended in dissociation buffer and loaded without heating. The protein band was visualized by soaking the gel in 4 M sodium acetate solution for 30 min and exposing the gel to light from a black box containing a circular lamp. The piece of gel containing the NCP (the gel remained transparent) was excised and homogenized in 0.02 M PB, pH 8.0, with a mortar and pestle to extract the NCP. Protein was precipitated by differential pH, saturated ammonium sulfate precipitation, and centrifugation. The final pellet was suspended in 1 mM PB, pH 8.0, and dialyzed against the same buffer overnight. The solution was stored at –20°C.

Serological assays for RGSV NCP. Antisera to NCP obtained from differential pH precipitation and NCP obtained from SDS-PAGE were produced by intramuscular injection to rabbits. Approximately 500 μg of each protein preparation was mixed with an equal volume of Freund's complete adjuvant and injected into the muscle of the rabbit's hind leg. Four weekly injections were made, and bleedings were done the fifth week. Serum was separated and stored at –20°C. Double immunodiffusion tests were done in 0.8% Difco agar containing 0.85% NaCl, 0.05% NaN_3, 0.01 M EDTA, and 0.15% SDS. Plates were incubated at room temperature and observed for 2 days.

DAS ELISA was done according to the procedure of Clark and Adams (3). The polystyrene plates (Immulon II, Dynatech Laboratories, Alexandria, VA) were coated with purified NCP IgG at 1 μg/ml in coating buffer (0.05 M sodium carbonate, pH 9.6). Test antigens were prepared in PBST, pH 7.4. Alkaline-phosphatase-conjugated IgG was used at 7 μg/ml. Indirect ELISA was done as described by Koenig (16). Microtiter plates were coated with test antigens in coating buffer. Anti-NCP IgG was used at 1 μg/ml in PBST, and alkaline-phosphatase-conjugated goat anti-rabbit IgG was used at 1/1000 dilution in PBST containing 2% PVP 40. For both DAS and indirect ELISA, plates were incubated with p-nitrophenyl phosphate substrate at 1 mg/ml in diethanolamine buffer. After 1-hr incubation, the absorbance at 405 nm was read with a Dynatech Minireader II.

RESULTS

Detection and purification of the RGSV NCP. Large amounts of needle-shaped crystals (1.8 to 9.6 μm long and 0.04 to 0.4 μm wide) were obtained by differential pH precipitation from leaves of RGSV-infected plants (Fig. 1). The protein crystals dissolved at a pH above 7.0 and recrystallized at a pH below 6.0. The crystals were composed of a single protein with a molecular mass of 24 kDa; the RGSV RNP has a molecular mass of 37 kDa (Fig. 2). The purified protein had a typical protein absorption spectrum with maximum absorbance at 278, minimum absorbance at 252, and an $A_{260/280}$ absorbance ratio of 0.6.

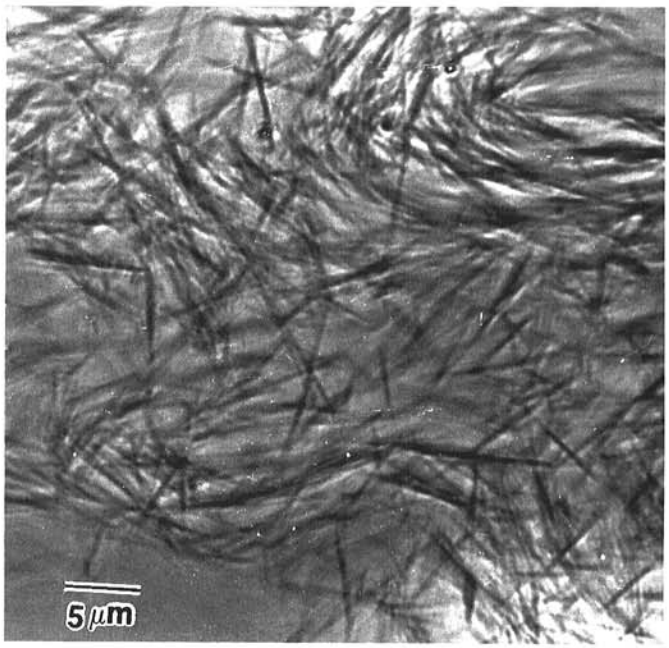

Fig. 1. Needle-shaped crystals in purified rice grassy stunt virus noncapsid protein as seen under phase contrast microscopy.

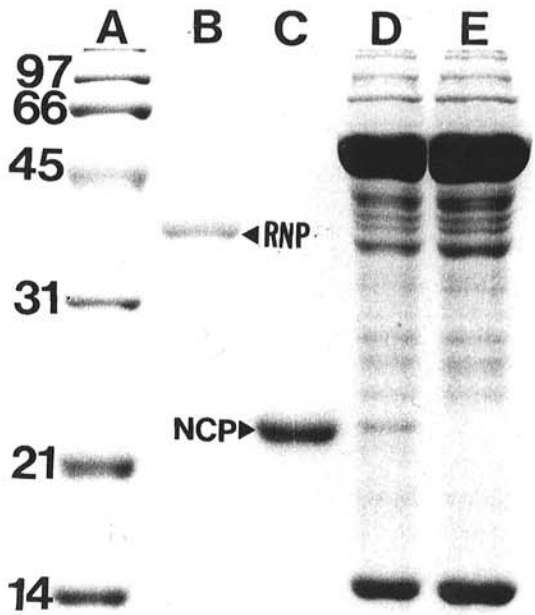

Fig. 2. Sodium dodecyl sulfate polyacrylamide slab gel (12%) showing purified 37-kDa rice grassy stunt virus (RGSV) ribonucleoprotein (RNP) and 24-kDa noncapsid protein (NCP). Lane A contains the protein standards, lane B the purified RGSV RNPs, lane C the purified RGSV NCP, lane D extracts from RGSV-infected rice leaves, and lane E extracts from healthy rice leaves.

Because of its similarity to other tenuiviruses, we will refer to this protein as the RGSV NCP. About 20 mg of protein was obtained per 100 g of RGSV-infected fresh tissue by the differential pH precipitation method. The RGSV NCP was easily detected by SDS-PAGE in extracts of leaves from RGSV-infected rice plants but not in leaves from healthy plants (Fig. 2). The antisera obtained from the NCP prepared by differential pH precipitation reacted strongly with sap from RGSV-infected rice plants and with purified RGSV in DAS ELISA tests, but not with sap from healthy plants. This result indicated that the NCP purified by differential precipitation was contaminated with RGSV RNP. Therefore, the NCP was further purified by SDS-PAGE. The SDS-PAGE-purified NCP retained its activity of recrystallization and was free of contamination with RGSV RNP.

The RGSV NCP antisera obtained from NCP prepared by differential pH precipitation and by SDS-PAGE were compared in Western blots. Both antisera detected the NCP in extracts of leaves from RGSV-infected rice plants (Fig. 3, lane D). When antiserum to the NCP purified by differential pH precipitation was used as a primary antibody, reactions were obtained to the RGSV NCP and the RGSV RNP (Fig. 3). Corresponding reactions were observed in extracts of leaves from RGSV-infected rice plants and viruliferous planthoppers. When antiserum to NCP purified by SDS-PAGE was used, however, reactions were obtained only to the RGSV NCP (Fig. 3). These results indicated that differential pH purification of NCP did not totally eliminate RGSV RNP, whereas RGSV RNP-free preparations were obtained by SDS-PAGE.

Western blots of viruliferous planthoppers showed negative reactions to NCP (Fig. 3, lane G), although RGSV was detected in individual and pooled viruliferous planthoppers by DAS and indirect ELISA with RGSV RNP antiserum. Likewise, NCP was not detected in planthoppers by either DAS or indirect ELISA using an antiserum to NCP purified by SDS-PAGE (data not shown).

Serological assays for RGSV NCP. Because a sensitive and accurate serological test for detecting RGSV-infected plants is needed, we compared the sensitivity of DAS and indirect ELISA methods using an antiserum to SDS-PAGE-purified NCP. Tenfold serial dilutions of the purified NCP, RGSV RNP, and leaves from RGSV-infected plants (where initial concentrations were adjusted to 1 mg/ml, A_{260} = 3.0, and 1 g of tissue per milliliter, respectively) were tested. The sap of infected leaves reacted at 10^{-1} dilution in DAS ELISA and up to 10^{-5} dilution in indirect ELISA (Fig. 4). Purified NCP was detected up to 10^{-4} dilution in DAS

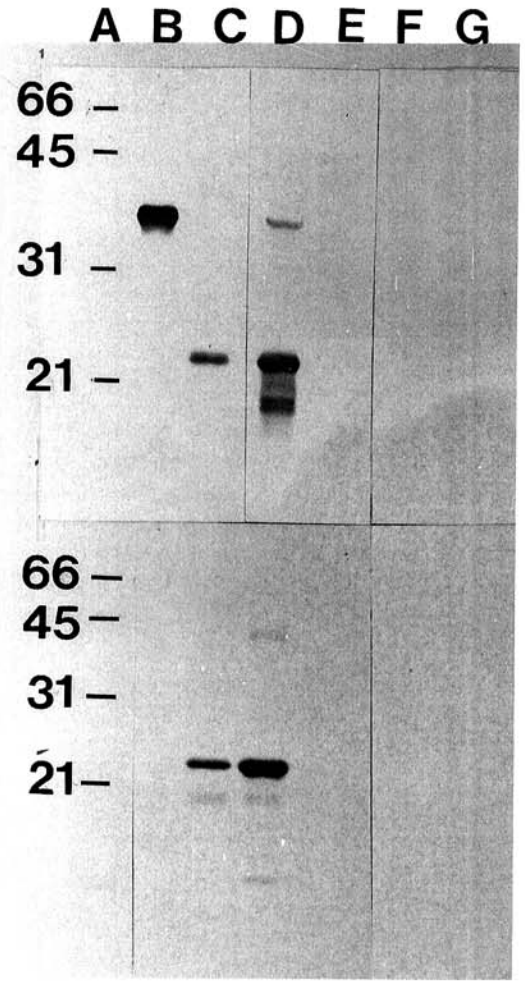

Fig. 3. Western blot analysis of ribonucleoprotein (RNP) of rice grassy stunt virus (RGSV) in plants and planthoppers. The upper blot was reacted with an antiserum to the noncapsid protein (NCP) purified by differential pH precipitation; the lower blot was reacted with an antiserum to RGSV RNP purified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Lane A is protein standards, lane B purified RGSV RNP, and lane C SDS-PAGE-purified NCP. Lane D is extracts from RGSV-infected rice leaves, lane E extracts from healthy rice leaves, lane F extracts from virus-free planthoppers, and lane G extracts from viruliferous planthoppers.

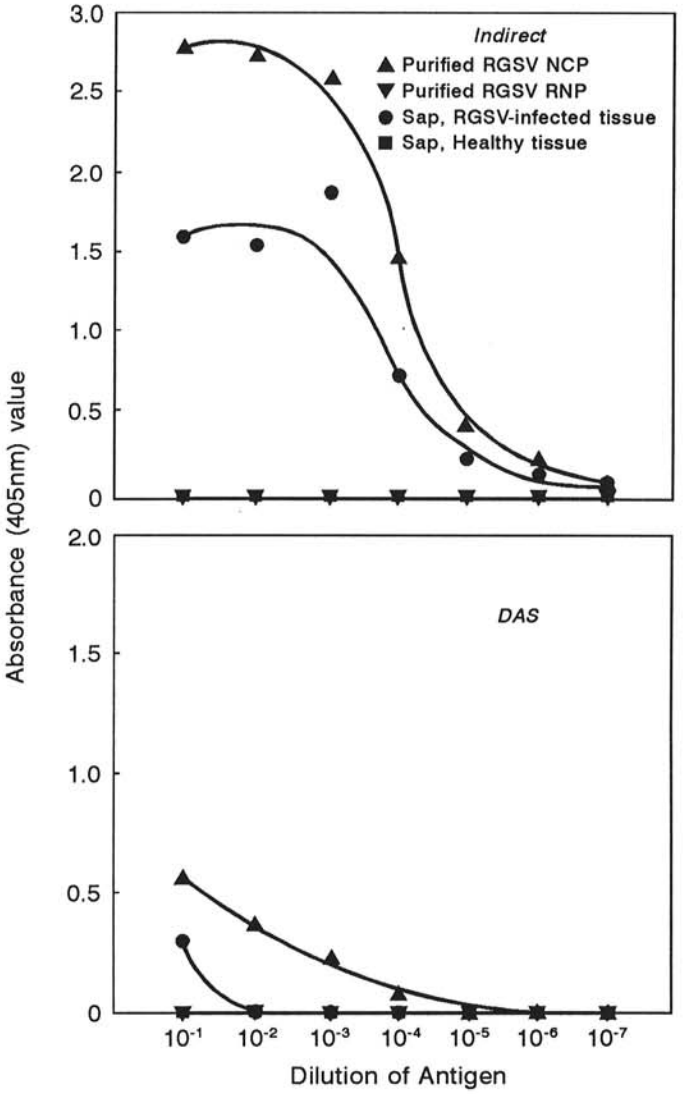

Fig. 4. Absorbance (405 nm) values for indirect and double-antibody sandwich (DAS) enzyme-linked immunosorbent assays done with antiserum to 24-kDa rice grassy stunt virus (RGSV) noncapsid protein (NCP) purified by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Purified NCP, RGSV ribonucleoprotein (RNP), and plant sap were adjusted to initial concentrations of 1 mg/ml, A_{260} = 3.0, and 1 g of tissue per milliliter, respectively.

ELISA and up to 10^{-5} dilution in indirect ELISA. These results showed that indirect ELISA was about 10 times more sensitive than DAS ELISA in detecting NCP in the sap from infected leaves and in purified NCP preparations. No reaction was obtained with sap from healthy leaves or with purified RGSV in either ELISA method.

In double immunodiffusion tests, the antiserum of RGSV NCP gave single precipitin lines against NCP of RSV and, reciprocally, the antiserum of RSV NCP also reacted with RGSV NCP.

DISCUSSION

The results of this study indicate that RGSV, like other members of the genus *Tenuivirus,* produces a protein that exhibits characteristic properties of tenuivirus NCP. The RGSV NCP is very similar to other tenuivirus NCPs but has a slightly higher molecular mass (24 kDa, compared to 20.5 kDa for RSV NCP [17], 19.8 kDa for MSpV NCP [12], and 20.1 kDa for RHBV NCP [24]). RGSV NCP and RSV NCP are serologically related. These findings further support the conclusion that RGSV belongs to the genus *Tenuivirus* and that production of NCP is a common property of tenuiviruses. However, the function of NCP is not known.

We and other workers (13) have detected tenuiviruses in their corresponding planthopper vectors by ELISA. However, we failed to detect NCP in planthoppers with ELISA and even with Western blot analysis. These results were similar to those reported for MSpV (6). In contrast, Toriyama (26) reported that RSV NCP was detected by ELISA in the planthopper vector. Our results showed that differential pH precipitation does not eliminate the corresponding tenuivirus RNP antigens and yields antiserum that reacted in ELISA with purified RGSV RNP. Thus, the NCP preparation used for antiserum production in the Toriyama study (26) may also have been contaminated with RSV RNP.

The results of this study represent an important development in serological studies on RGSV because they make possible the use of antisera to the NCP produced by RGSV-infected plants. Unlike RGSV RNPs, whose preparation is laborious, NCP is easy to purify. Moreover, a sufficient amount of NCP is produced from small quantities of infected tissues. The RGSV NCP antisera obtained from the differential precipitation method can be applied for routine indexing of RGSV-infected plants. The RGSV NCP antibody obtained by SDS-PAGE can be applied to detect RGSV NCP specifically in infected plants.

LITERATURE CITED

1. Atabekov, J. G., Popova, G. A., Kiselev, N. A., Kaftanova, A. S., and Petrovsky, G. V. 1968. In vitro polymerization of winter wheat mosaic virus antigen. Virology 35:458-472.
2. Burnette, W. N. 1981. "Western blotting": Electrophoretic transfer of proteins from sodium dodecyl sulfate polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated Protein A. Anal. Biochem. 112:195-203.
3. 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. 48:311-317.
4. Falk, B. W., Morales, F. J., Tsai, J. H., and Niessen, A. J. 1987. Serological and biochemical properties of the capsid and major non-capsid proteins of maize stripe, rice hoja blanca, and *Echinochloa* hoja blanca virus. Phytopathology 77:196-201.
5. Falk, B. W., and Tsai, J. H. 1983. Assay for maize stripe virus-infected plants by using antiserum produced to a purified non-capsid protein. Phytopathology 73:1259-1262.
6. Falk, B. W., Tsai, J. H., and Lommel, S. A. 1987. Differences of levels of detection for the maize stripe virus capsids and major non-capsid proteins in plant and insect hosts. J. Gen. Virol. 68:1801-1811.
7. Francki, R. I. B., Fauquet, C. M., Knudson, D. L., and Brown, F. 1991. Classification and Nomenclature of Viruses. Fifth Report of the International Committee on Taxonomy of Viruses. Arch. Virol. Suppl. 2. Springer-Verlag, New York.
8. Gingery, R. E. 1988. The rice stripe virus group. Pages 297-329 in: The Plant Viruses, Vol. 4: The Filamentous Plant Viruses. R. G. Milne, ed. Plenum Press, New York.
9. Gingery, R. E., Nault, L. R., and Bradfute, O. E. 1981. Maize stripe virus: Characteristics of a member of a new virus class. Virology 112:99-108.
10. Hibino, H. 1986. Rice grassy stunt virus. Descriptions of Plant Viruses, No. 320. Commonwealth Mycological Institute and Association of Applied Biologists, Kew, Surrey, England.
11. 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.
12. Huiet, L., Klaassen, V., Tsai, J. H., and Falk, B. W. 1990. Identification and sequence analysis of the maize stripe virus major noncapsid protein gene. Virology 179:862-866.
13. Iwasaki, M., Nakano, M., and Shinkai, A. 1985. Detection of rice grassy stunt virus in planthopper vector and rice plants by ELISA. Ann. Phytopathol. Soc. Jpn. 51:450-458.
14. Kakutani, T., Hayano, Y., Hayashi, T., and Minobe, Y. 1990. Ambisense segment 4 of rice stripe virus: Possible evolutionary relationship with phleboviruses and uukuviruses (Bunyaviridae). J. Gen. Virol. 71:1427-1432.
15. Kiso, A., and Yamamoto, T. 1973. Infection and symptom development in rice stripe disease, with special reference to disease-specific protein other than virus. Rev. Plant Prot. Res. 6:75-100.
16. Koenig, R. 1981. Indirect ELISA methods for the broad specificity detection of plant viruses. J. Gen. Virol. 55:53-62.
17. Koganezawa, H. 1977. Purification and properties of rice stripe virus. Trop. Agric. Res. Ser. 10:151-154.
18. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T-4. Nature (London) 227:680-685.
19. Miranda, G. J., and Koganezawa, H. 1992. Variants of rice grassy stunt (RGSV) in the Philippines. Int. Rice Res. Notes 17(2):24-25.
20. Murphy, F. A., Fauquet, C. M., Bishop, D. H. L., Ghabrial, S. A., Jarvis, A. W., Martelli, G. P., Mayo, M. A., and Summers, M. D. 1995. Virus Taxonomy: Sixth Report of the International Committee on Taxonomy of Viruses. Arch. Virol. Suppl. 10. Springer-Verlag, New York. 586 pp.
21. Ou, S. H. 1985. Rice Diseases, pp. 45-48. Commonwealth Mycological Institute, Kew, Surrey, England.
22. Pelligrini, S., and Bassi, M. 1978. Ultrastructure alterations in rice plants affected by "grassy stunt" disease. Phytopathol. Z. 92:247-250.
23. Ramirez, B. C., and Haenni, A. L. 1994. Molecular biology of tenuivirus, a remarkable group of plant viruses. J. Gen. Virol. 75:467-475.
24. Ramirez, B. C., Iozano, I., Constantino, L. M., Haenni, A. L., and Calvert, L. A. 1993. Complete nucleotide sequence and coding strategy of rice hoja blanca virus RNA4. J. Gen. Virol. 74:2463-2468.
25. Sta. Cruz, F. C., and Koganezawa, H. K. 1987. Cellular inclusions in rice grassy stunt virus (RGSV)-infected rice. Int. Rice Res. Notes 12(6):23.
26. Toriyama, S. 1986. Rice stripe virus: Prototype of a new group of viruses that replicate in plants and insects. Microbiol. Sci. 3:347-351.