A Novel Detection and Identification Technique for Plant Viruses: Rapid Immunofilter Paper Assay (RIPA)

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ABSTRACT

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A rapid immunofilter paper assay (RIPA) for cucumber mosaic virus (CMV) or tobacco mosaic virus (TMV) was developed using latex beads coated with antibodies to either virus. White latex-coated antibody was immobilized as a solid in a line on the surface near the bottom of a filter paper strip. The bottom end of this paper strip was dipped for 3 min into a mixture of leaf extract from an infected plant and dyed-latex coated with the virus antibody. A colored band appeared on the line where the white latex had been immobilized. With RIPA, as little as 5 ng/ml of purified TMV or 50 ng/ml of purified CMV could be detected with the naked eye. In the extract from infected tobacco plants, TMV was detected to a dilution of 10^{-7}, and CMV was detected to a dilution of 10^{-5} with the naked eye. The conditions for coating antibody to the latex beads were best at pH 7.2 using Tris-buffered saline and a 100-μg/ml concentration of antibody. When RIPA was applied to the extract of infected plants of 13 species in six families, CMV was detected from all species. When filter paper strips were measured by chromatoscanner, each sample virus could be detected at concentrations 10 to 100 times lower than concentrations detected with the naked eye. The sensibility of RIPA measured by chromatoscanner was similar to sensibilities of ELISA tests for CMV.

The application of serological methods for the detection of plant viruses in the field or for epidemiological studies requires the development of rapid, simple, and sensitive immunoassays. Antigen-antibody reactions are generally either reactions of sedimentation, agglutination, or labeled antibodies. Of these, the Ouchterlony immunodiffusion technique; the passive hemagglutination assay using sheep red blood cells, artificial gelatin or latex beads; and the enzyme-linked immunosorbent assay (ELISA), radio immunoassay, and fluorescence immunoassay commonly have been used to detect plant viruses. The above methods are not always suitable for field diagnosis because they require specialized equipment and are time-consuming.

We have developed a rapid immunofilter paper assay (RIPA) that can easily be used for virus disease diagnosis with considerably high sensibility. In this paper, we demonstrate the use of RIPA for the detection of cucumber mosaic virus (CMV) or tobacco mosaic virus (TMV).

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MATERIALS AND METHODS

Virus and maintenance. CMV-C (serotype Y) (8) and TMV-T (11) were mechanically inoculated to *Nicotiana tabacum* L. 'Xanthi' using 0.1 M phosphate buffer, pH 7.0, containing 0.01 M Na_2SO_3. Plants for inoculation were grown in a glasshouse maintained at 20–30 C. Purification of CMV and TMV was performed according to conventional methods (8,11). Purified virus was diluted with Tris-buffered saline (TBS, containing 0.02 M Tris and 0.15 M NaCl and adjusted to pH 7.2 with HCl), and virus concentrations were estimated using extinction coefficients at 260 nm of 5 for CMV and 3 for TMV by spectrophotometer. Purified viruses then were used for titration. Leaf extract was prepared by grinding leaves with a pestle and mortar together with an equal amount of TBS containing 0.01 M Na_2SO_3 (w/v). In CMV, serologically related strains showing spur in agar gel diffusion tests also were used for comparison. They were a leguminous strain (12) of serotype Y, and SR (13) or P (4) strains of serotype P.

Antibody preparation. Antiserum to CMV-Y (10) was used for CMV detection, and antiserum to TMV-T (11) was used for TMV detection. Either antiserum was applied to a DEAE-cellulose SH column, and antibody was isolated as IgG fractions eluted from antiserum with 0.02 M phosphate-buffered saline.

Coating of antibody to latex. Two kinds of latex beads (Japan Synthetic Rubber Co. Ltd., Tsukuba) were used for RIPA. Non-dyed latex (white) was used for the solid phase, and dyed (pink) latex was used for the tracer. For coating of antibody, white latex was diluted to 0.5% concentration (v/v) with TBS, and pink latex was diluted to 1% concentration (v/v) with TBS. These then were mixed with the same volume of IgG solution (Fig. 1A). The suitable antibody concentration for coating to both latexes was examined at concentrations between 100 and 500 μg/ml of antibody. The pH of TBS for coating antibodies on latex beads was examined at pH 6.2, 7.2, or 8.2. The mixtures were incubated at room temperature for 2 hr with occasional shaking. The latex beads coated with antibody were washed three times by centrifugation at 15,000 rpm for 10–15 min with TBS-BSA (TBS with 0.1% [w/v] bovine serum albumin), and the precipitates were resuspended in TBS-BSA at 0.5% (v/v) in the case of white latex or 1% (v/v) for pink latex. The latexes were stable at 4 C for more than 6 mo.

Assay procedure for RIPA. The procedure for the RIPA was modified from Bangs (1). Five microliters of white latex coated with antibody to each virus was applied approximately 1.5 cm from the lower end of a 8 × 0.5 cm strip of Whatman glass filter paper. The immobilized filter paper strips were kept in a plastic desiccator at room temperature after air-drying. These dried filter paper strips can be stored at room temperature for more than 12 mo. Pink latex was diluted to 0.025% (v/v) with TBS and was mixed with an equal volume of either purified virus solution or leaf extract. Tween 20 then was added to the mixture at the final concentration of 0.3% (v/v). The filter strip was wetted by dipping it about 0.5 cm from the end of the filter paper strip into the mixture of latex solution and leaf extract. After a few minutes from the start of dipping, a pink band appeared on the immobilized white latex. The procedure is illustrated in Figure 1B.

Quantitative measurement of signals. The dried filter paper strip was photographed and printed by auto mode (f-stop = 8). Quantitative estimation of signals was done by scanning the processed

photographs with a Shimadzu chromatoscanner with visible light at 700 nm. The measured values were expressed as relative values of absorbance after subtraction of values measured at a nonreacted area, just above (0.3–0.5 cm) the band showing signals, on the same filter paper strip.

Comparison between RIPA and ELISA. The ELISA tests for comparison with RIPA were done according to the method described by Clark and Adams (2) with the same samples of CMV, either purified virus or sap of infected tobacco leaf. The absorbance of OD_{410} after incubation time over 1 hr from the addition of substrate was measured with a microplate reader.

RESULTS

Condition for antibody coating. The suitable antibody concentration for coating on both latexes was 100 μg/ml. In the variation of pH for coating antibodies on latex, pH 7.2 exhibited the best signal on the filter paper strip. Therefore, all latexes were coated with antibodies at 100 μg/ml and pH 7.2 for the following experiments.

Assay by RIPA. When the immobilized filter paper strips were dipped into the mixture of pink latex and purified virus (CMV or TMV), the pink latex moved by capillary action, and the flow reached to the immobilized zone. A few minutes later, a pink-colored line was formed on that zone (arrow in Fig. 2). Although drying of the strip was not necessary for the visual inspection of RIPA results, drying and storage at room temperature for more than 6 mo induced no sign of color fading on the tested strip. When the leaf extracts of infected tobacco plants were applied, the dipped filter paper strip also showed the pink band on the immobilized zone, together with the green color of the leaf extract at the dipping phase. The CMV system of RIPA never reacted to the samples containing TMV and vice versa, as this system depends only on antigen-antibody reactions. These reactions were easily inhibited by adding the same antibody coated to latex to the mixture of the sample and pink latex, which demonstrates an inhibitory effect for RIPA by the antibody. When buffer alone or healthy plant extract was used, no pink band appeared. All RIPA results tested so far were reproducible.

Sensitivity of RIPA. The filter paper strips showed a positive reaction to 500 ng/ml CMV, forming a pink line by antigen-antibody reaction (Fig. 2, arrow). This signal became weaker as the concentration of CMV antigen decreased. At 50 ng/ml, CMV was still detectable, whereas there was no visible detectable signal at 10 ng/ml. When purified TMV was tested, the signals were visible until 5 ng/ml. This sensitivity of RIPA was not affected when healthy tobacco sap was used as a buffer for the dilution of purified viruses. When the extract of infected tobacco leaves was used for RIPA, TMV was detected down to a dilution of 10^{-7}, and CMV was detected down to a dilution of 10^{-5} with the naked eye. Furthermore, when photographs of the filter paper strips were measured by chromatoscanner, these reactions could be detected until 5 ng/ml for CMV and 1 ng/ml for TMV. When the extract of infected leaves was used, the signals were detected down to a dilution of 10^{-6} for CMV and 10^{-8} for TMV (Figs. 3 and 4). The determination of the sensitivity of RIPA was tested three times against either infected leaves or purified viruses.

In some serological tests, such as double-sandwich ELISA, the sensitivity of detection was reduced when a different serotype of the virus was used (7). To check this drawback for routine indexing, two CMV isolates belonging to the different serotype of P from CMV-C were used for comparison. These two isolates were detected by RIPA to the same dilution (*data not shown*), such as in indirect ELISA.

Tests for nonspecific reaction of RIPA. CMV was easily detected by RIPA from the following plant species infected by CMV. Each infected leaf of 13 species from six families was prepared as sap of leaf according to the RIPA assay

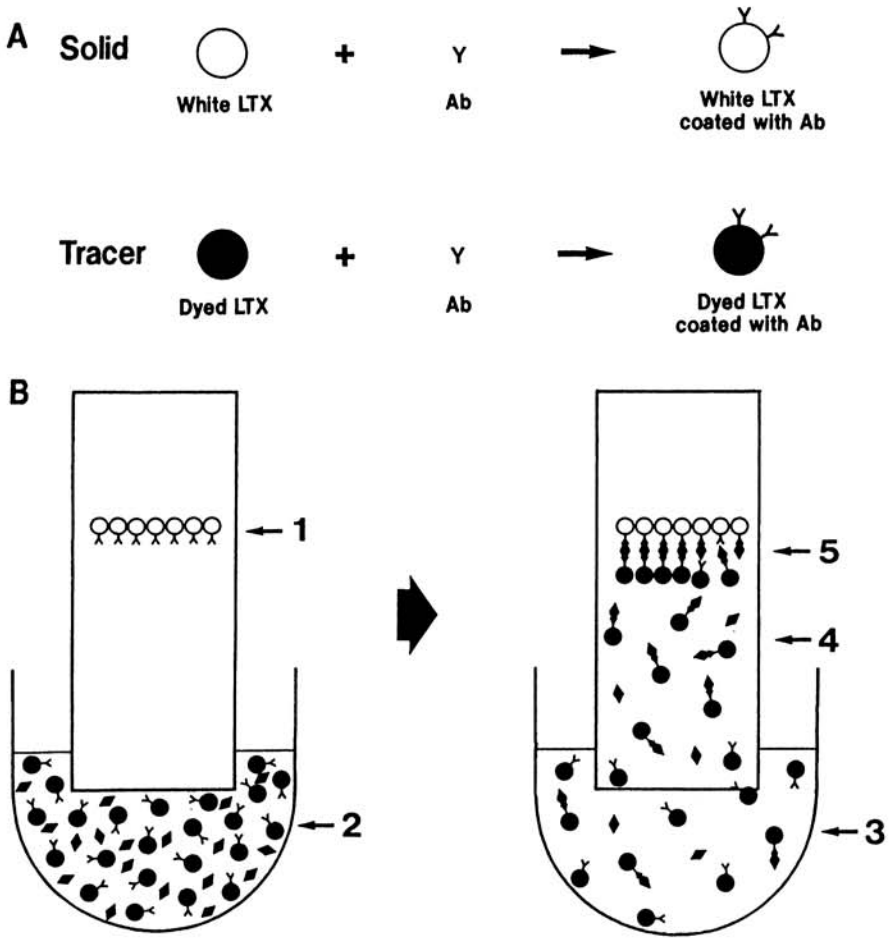

Fig. 1. RIPA test using dyed latex (pink). **(A)** Preparation of latex (LTX) coated with antibody (Ab) for solid and tracer; **(B)** principle of the RIPA technique. 1 = Immobilized white latex coated with antibody as a solid in a line on the surface of the filter paper strip. 2 = Bottom end of this strip is wet with the mixture of both sample and dyed latex coated with antibody as the tracer. 3 = Virus (diamond-shaped particles) reacts with dyed latex coated with antibody. 4 = Complex flow moves by capillary action. 5 = Virus on dyed latex is sandwiched with immobilized white latex coated with antibody and forms a pink line.

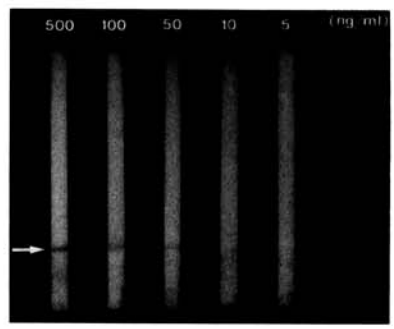

Fig. 2. Sensitivity of detection for purified CMV using RIPA. Serial dilutions of purified CMV were assayed at concentrations of 500, 100, 50, 10, and 5 ng/ml. Pink lines (arrow) were formed on filter paper strip until 50 ng/ml of CMV by judgment with the naked eye.

procedure. The plants included *Spinacia oleracea* L. and *Beta vulgaris* L. var. *cicla* (L.) W. Koch from the Chenopodiaceae; *Raphanus sativus* L. var. *hortensis* Backer and *Brassica rapa* L. var. *amplexicaulicaulis* Tanaka & Ono 'Pe-tsai' from the Cruciferae; *Lycopersicon esculentum* Mill., *N. tabacum* L., and *N. glutinosa* from the Solanaceae; *Cucumis sativus* L., *C. melo* L., and *Cucurbita moschata* (Duchesne) Duchesne ex Poir. from the Cucurbitaceae; *Zinnia elegans* Jacq. and *Lactuca sativa* L. from the Compositae; and *Zea mays* L. from the Gramineae. RIPA did not show any reactions for dyed latex coated with native antibody from normal rabbit serum (preimmune serum), buffer only, or healthy plant extracts of 26 species in nine families, including the plants described above.

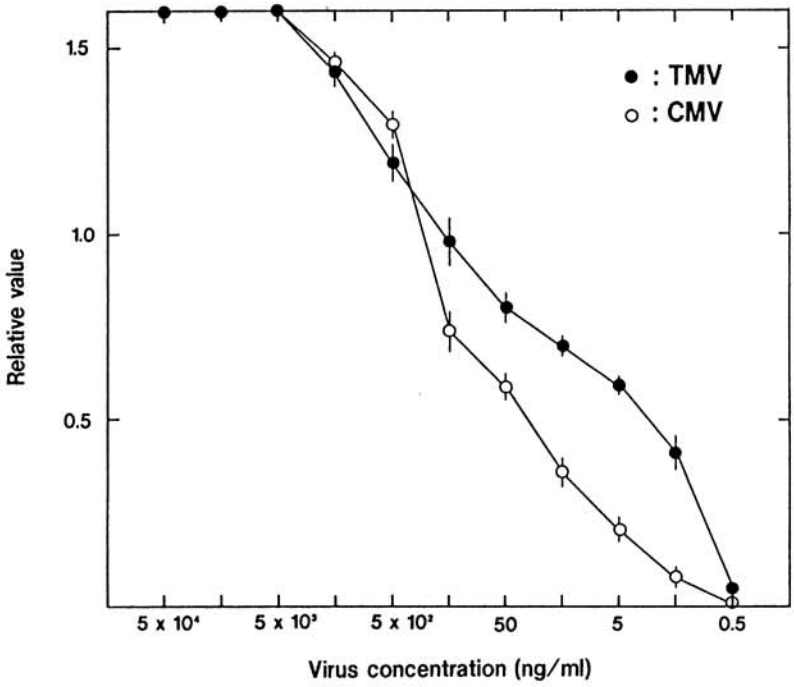

Fig. 3. Quantitative estimations of RIPA for purified CMV or TMV. Relative values were measured absorbance at visible light (700 nm) after subtraction of values of a nonreacted filter paper strip by chromatoscanner. The hash marks represent the range of standard deviation.

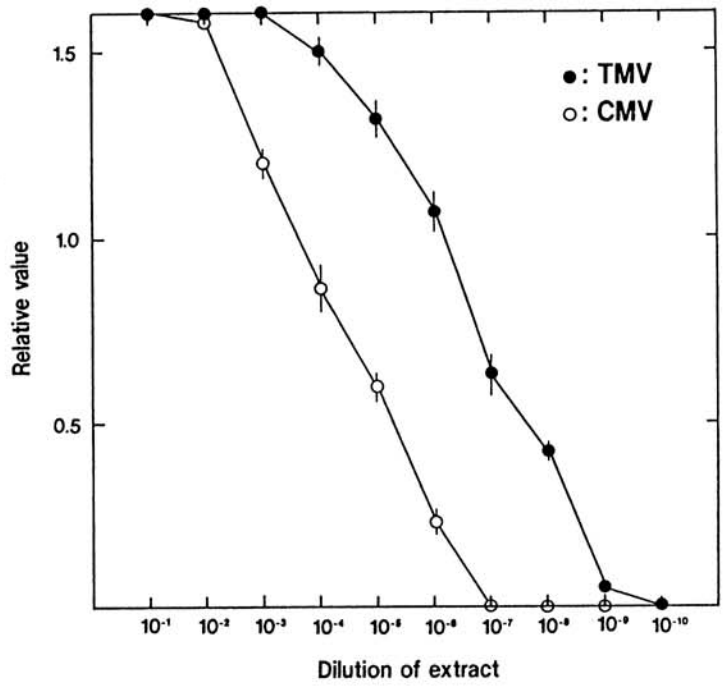

Fig. 4. Quantitative estimation of RIPA using the extracts of infected tobacco plants with either CMV or TMV. Relative values were measured absorbance at visible light (700 nm) after subtraction of values of a nonreacted filter paper strip by chromatoscanner. The hash marks represent the range of standard deviation.

Comparison between RIPA and ELISA. The end point of sensitivity between RIPA and ELISA was compared with identical samples of either purified CMV or sap of CMV-infected tobacco leaf. These results are presented in Figures 5 and 6. By ELISA tests, positive reaction was detectable at 10 ng/ml for purified virus and at dilutions of 10^{-5} of leaf sap of infected tobacco with the naked eye. With the use of the microplate reader, positive reactions were detected at as little as 5 ng/ml for purified virus or a 10^{-6} dilution for infected sap by measurement. In RIPA tests, the sensitivity was similar to the sensitivity with ELISA. Consequently, the sensibility of RIPA was considered to be almost the same as that of ELISA.

DISCUSSION

We have developed a new, simple, rapid, sensitive and virus-specific detection technique called RIPA. RIPA is a landmark technique for plant virus disease diagnosis. The filter paper strips coated with antibody can be stored at room temperature for more than 1 yr in a desiccator. This enabled us to prepare a batch of strips at one time and store them for future use. The coated strips can be used as easily and simply as pH test paper. A technique using dyed latex particles was applied for a human pregnancy test in 1988 (1). In animal diseases, this technique has been used for the detection of chlamydia (1). No other home tests have surfaced to date. So far, no reliable and sensitive filter paper method such as RIPA has been successfully applied for virus diagnosis.

ELISA is used widely in the diagnosis of plant viruses (2), but it requires laboratory equipment. Until now, techniques reported as rapid diagnosis systems were practical applications of ELISA that depended on the reaction of an enzyme, by exchanging a 96-well polyethylene plate to a nitrocellulose filter or chromatography paper. These techniques require many steps and complicated operations (3,5,9). Hsu (6) described a detection technique based on an agglutinative reaction using both nitrocellulose paper and colloidal gold, but that technique is an application of western or dot blotting analyses, which are more difficult and time-consuming than simple chromatographical tests, such as RIPA. Moreover, with RIPA, no skill or experiences is needed to detect results easily within 5 min with the naked eye, and the results also can be measured with a chromatoscanner, if higher sensitivity or quantitative analysis is necessary. With a chromatoscanner, viruses can be detected at concentrations 10 to 100 times lower than concentrations detected by the naked eye. The end points of sensitivity of RIPA were similar to

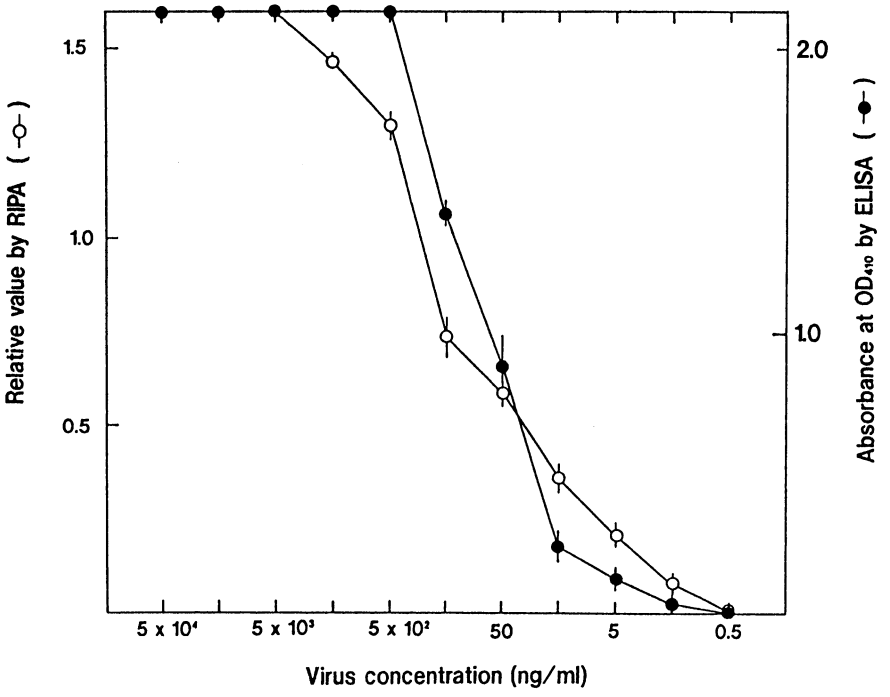

Fig. 5. Comparison of sensitivity for detection of purified CMV between RIPA and ELISA. Each data point represents the average ELISA reading of three tests. The hash marks represent the range of standard deviation.

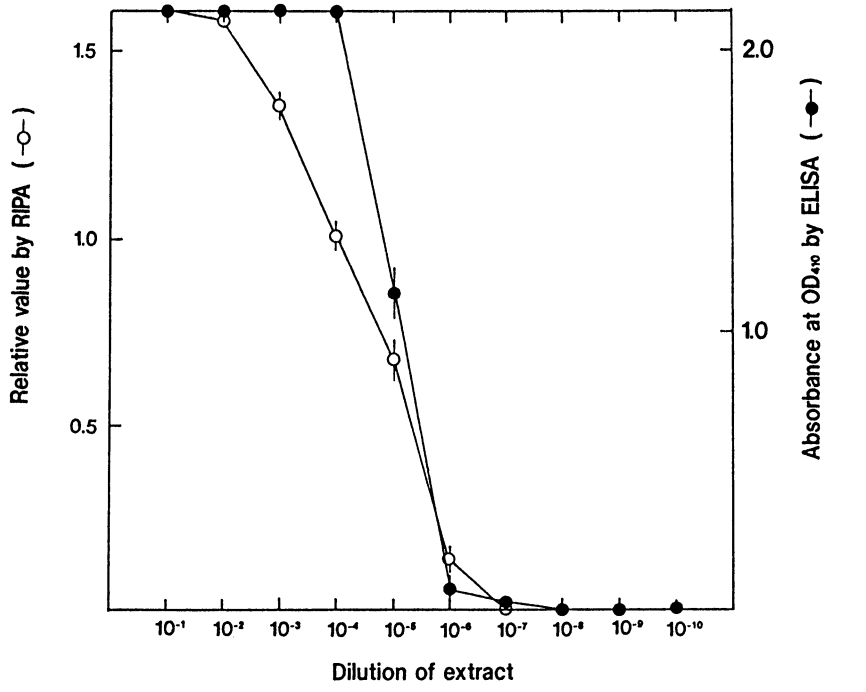

Fig. 6. Comparison of sensitivity for detection from sap of tobacco leaf infected with CMV between RIPA and ELISA. Each data point represents the average ELISA reading of three tests. The hash marks represent the range of standard deviation.

ELISA tests using both the same antibody and antigen. The sensibility of RIPA was demonstrated by these results. Although viruses with spherical and rodlike particles, CMV and TMV, were examined as materials in this work, the usefulness of RIPA for the detection of viruses with elongated particles, such as PVY, has yet to be tested.

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