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Improvement of Antigen Blotting in a Tissue Blot Immunobinding Assay for the Detection of Two Chili Pepper Viruses

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Abstract The tissue blot immunobinding assay (TBIA) is widely used for the detection and localization of plant viruses in various plant tissues. The basic experimental procedures of TBIA sampling and blotting were simplified using commercially available micropipette tips. This method was termed the ring-blot immunobinding assay (R-BIA), as the blot on the membrane forms a ring shape. The detection efficacy of R-BIA was tested for two chili pepper viruses, pepper mild mottle tobamovirus (PMMoV) and pepper mottle potyvirus (PepMoV), following the optimized serological procedures of TBIA (length of the incubation period and BSA concentration, and primary and secondary antibodies). Sensitivity of the R-BIA was about 1 ng/ml of purified PMMoV in pepper leaf sap from a healthy pepper plant. R-BIA also showed high specificity in the detection of PMMoV and PepMoV. Moreover, the modified sampling and blotting procedures were simpler and more reliable than other TBIA methods (such as whole-leaf blotting and crushed-leaf blotting), suggesting that the R-BIA may be used for medium- to large-scale detection of plant viruses in laboratories with minimal facilities.

Keywords: Chili pepper, detection, pepper viruses, ring-blot, tissue blot immunobinding assay

Serological techniques provide useful tools for detecting plant viruses as well as other microbes owing to the ease with which they can be performed and their reliable levels of sensitivity and specificity [8, 10, 11, 14, 15, 19, 22]. The most frequently used technique is the enzyme-linked immunosorbent assay (ELISA) [1, 3, 7, 14, 18]. Automation of ELISA has enabled seed companies and plant verification offices to perform large-scale diagnosis of various plant viruses. As an alternative to ELISA, PCR-based diagnostic techniques such as RT-PCR [4, 16, 17] and mutiplex PCR [9] have been developed and can be used to detect plant viruses with a high degree of sensitivity and diagnostic capacity. However, PCR-based techniques generally have some drawbacks, such as the labor-intensive and time-consuming procedures required for preparing the viral antigen and RNA/DNA templates from various plant tissues. They also require chemicals and machines that can be too expensive for routine laboratories to afford.

The tissue blot immunobinding assay (TBIA) is another method for the rapid and sensitive diagnosis of plant viruses, which is comparable to the above techniques. It is particularly useful for detecting viral distribution over whole plants [1, 12, 13]. Moreover, it costs much less than ELISA or PCR-based techniques. Its drawbacks, however, lie in the difficulty of automation and its limited application to a large-scale diagnostic procedure, largely because of relatively onerous sample preparations compared with ELISA and PCR. Improvements in sample preparation are required for TBIA to be developed into a high-throughput screening tool for the diagnosis of plant viruses. The method described in this paper, termed the ring-blot immunobinding assay (R-BIA), is a procedure in which sample preparations for antigen extraction and deposition are simplified to meet this requirement.

Evaluation of R-BIA was made using two chili pepper viruses from two important plant viral groups: the pepper mottle virus (PepMoV), a Potyvirus, and the pepper mild mottle virus (PMMoV), a Tobamovirus. Pepper plants (*Capsicum annuum* cv. Chilsungcho) infected with PepMoV isolate SNU1 [6] and PMMoV isolate 15 (I15) [5] were used as viral antigens. Optimization of serological conditions for R-BIA (antisera titers) followed the usual TBIA procedures, except that antigen preparation and deposition on the membrane filter were performed at 35°C in a shaking incubator at 45 rpm. Immunoglobulin G (IgG) as primary antibody was isolated from PMMoV [5] and

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PepMoV (ATCC PV-AS-889) polyclonal antibodies using Sepharose CL-4B and PD-10 columns according to the manufacturer's instructions (Pharmacia, Uppsala, Sweden). Nitrocellulose (NC) membrane with a pore size of 0.45 μm (Sigma-Aldrich, St. Louis, MO, U.S.A.) was equilibrated with 0.01 M Tris-buffered saline (TBS), pH 7.4, for 10–30 min. The wetted membrane was laid on Whatman No. 1 filter paper with appropriate moisture. The blotted membrane was washed twice in TBS containing 0.5% Tween-20 (TBST) for 10 min and then transferred into 20 ml of bovine serum albumin (BSA) solution for 60 min. The membrane was then incubated with primary antibody diluted in TBS for 2 h, washed twice, and incubated with secondary antibody (1 μg/ml) (that was an anti-rabbit IgG-alkaline phosphatase antibody produced in goat and available from Sigma-Aldrich) in TBS for 90 min. Before color development, the membrane was washed twice, and then immersed in an alkaline phosphatase substrate solution prepared by dissolving Sigma Fast BCIP/NBT (5-bromo-4 chloro-3 indolyl phosphate/Nitroblue tetrazolium) tablets (Sigma-Aldrich, St. Louis, MO, U.S.A.).

To optimize TBIA for the detection of each virus in pepper leaves, healthy and diseased leaves were rolled into a tight cone, cut manually using a razor blade, and subjected to blotting on slips of NC membrane. Each slip had five repeated blots that were tested individually to determine the optimum reaction conditions. The optimal conditions for removing nonspecific antibodies against PMMoV and PepMoV were 90- and 60-min incubation of purified IgG from the primary antisera with 20× diluted supernatant of healthy leaf extracts, respectively (Table 1). Appropriate concentrations of BSA solution, primary antibody, and secondary antibody were also determined as shown in Table 1: for PMMoV, 90 min of washing with 2% BSA, 60 min of incubation with 1 μg/ml primary antibody, and 60 min of incubation with 1 μg/ml secondary antibody; and for PepMoV, 60 min of washing with 1% BSA, 60 min of incubation with 0.5 μg/ml primary antibody, and 60 min of incubation with 1 μg/ml secondary antibody.

Using the conditions described above, R-BIA was compared with various TBIA methods using PepMoV, as it has generally been accepted that potyviruses are distributed at low concentrations in their hosts, which can prevent accurate diagnosis in some plants [21]. Currently, no information is available regarding the concentration and distribution of PepMoV in leaf tissues, limiting direct application of TBIA for PepMoV detection. Four sampling and blotting methods were used to evaluate the potential use of TBIA for detecting PepMoV. These methods required the whole leaf, serial transverse leaf sections, cross-sections of the rolled leaf, and leaf tissue that was crushed using a glass rod. For whole-leaf blotting, the adaxial surface of a virus-infected pepper leaf was transversely blotted by applying even pressure with a piece of Styrofoam pad. For leaf-roll blotting, a leaf was first rolled into a tight cone and then cut transversely with a new blade and blotted by gently pressing the newly cut surface onto the membrane. The crushed-leaf blot was prepared by depositing the part of the leaf surface that had been smashed with a glass rod onto the membrane. For R-BIA, the leaf surface was smashed 3–5 times with the larger open circle of a pipette tip (200-μl standard yellow pipette tip), immediately followed by pressing the tip wetted with the extract once on a NC membrane to make blots. The blots were developed using the optimal reaction conditions described in Table 1.

Table 1. Optimal additive concentrations and reaction times for a tissue blot immunobinding assay (TBIA)[a] to detect pepper mild mottle tobamovirus (PMMoV) and pepper mottle potyvirus (PepMoV) in pepper leaves.

Treatment (unit)	Optimal		Range tested
	PMMoV	PepMoV	
Absorption of nonspecific antibodies[b]			
Reaction time (min)	90	60	30, 60, 90
BSA solution			
Concentration (%)	2	1	1, 2, 3
Reaction time (min)	80	80	40, 80, 120
Primary antibody			
Concentration (μg/ml)	1	0.5	0.5, 1, 2, 4
Reaction time (min)	60	60	30, 60, 90, 120
Secondary antibody (1 μg/ml)			
Reaction time (min)	60	90	30, 60, 90

[a]Healthy (as negative controls) and diseased (as positive controls) leaves infected with each virus were rolled into a tight cone and sectioned manually using a razor blade. The freshly cut surface of the sections was blotted onto slips of nitrocellulose membrane. The slips were tested separately to determine the optimum reaction conditions (examining negative and positive color reactions and background tint).

[b]Healthy leaf extract for nonspecific antibody adsorption was prepared by grinding pepper leaves (1 g) in TBS (1 ml), centrifuging the extract at 8,000 rpm for 10 min, and diluting the supernatant 20-fold in TBS. Purified IgG as primary antibody from antisera against each virus was incubated with equal volume of the diluted supernatant. These mixtures were centrifuged at 8,000 rpm for 10 min. The supernatants were then used to determine the nonspecific antibody adsorption time to test the specificity of TBIA.

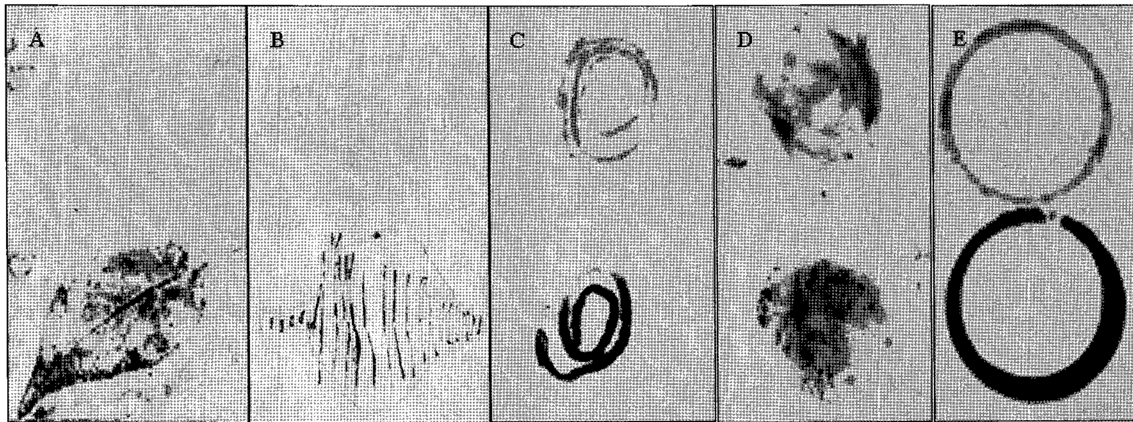

Fig. 1. Comparison of various blotting methods for analyzing the presence and distribution of pepper mottle potyvirus (PepMoV) in pepper leaf tissue.

Viral extract saps were blotted on PVDF membranes by hand with gentle pressure on the whole leaf (whole-leaf blot: **A**), serial transverse sections (serial transverse section blot: **B**), cross-section of a rolled leaf (rolled-leaf blot: **C**), leaf crushed using a glass rod (crushed-leaf blot: **D**), and open circle by pressing the larger open side of a commercially available micropipette tip wetted with each leaf extract (ring-blot [R-BIA]: **E**). Negative controls are shown in the top row. Blots were reacted in the optimal serological conditions described in Table 1.

The results of TBIA performed with the whole leaf and serial transverse sections revealed the distribution of PepMoV, and showed that in this case it was distributed throughout the entire leaf (Figs. 1A and 1B). Viral presence, but not distribution, was determined from the TBIA blots performed with the cross-section of the rolled leaf, blots from the crushed leaf, and by R-BIA (Figs. 1C, 1D, and 1E). Considering these aspects only, TBIA methods using the whole leaf or transverse sections seem to provide the most useful information (virus distribution) and have higher potential for viral detection than the other sampling methods tested, because the former use whole leaf tissues. However, on a large scale (*e.g.*, >1,000 samples), TBIA using the whole leaf or serial transverse sections is expensive, cumbersome, and time-consuming owing to the preparation of media, samples, and blots that require a large quantity of expensive NC membrane and serological reagents. When dealing with a large quantity of samples by such blotting methods, there is also a high potential for nontarget viral contamination. Other TBIA methods, such as leaf roll and crushed-leaf blotting were not as cumbersome or tedious as the two previous methods in terms of sample preparation; however, leaf-roll blotting, which is the most prevalent method in TBIA, is inefficient, as rolling the samples and changing razor blades is time consuming. Using the crushed-leaf blot method also has the same difficulties of sample deposition on the membrane as with the whole-leaf blot, although antigen extraction was relatively easier. In contrast, ring blotting enabled rapid and simple deposition of the sample on the membrane, as the use of a yellow micropipette tip provides a simple method to extract enough antigen without requiring special attention for sample deposition. For ring blotting, the addition of a drop of TBS buffer (*ca.* 3–5 μl) on the test leaf helps both easy antigen extraction and antigen binding to the circular surface of the yellow micropipette tip, to generate the complete formation of antigen blots. A positive antigen-antibody reaction in the ring blot (R-BIA) developed a purple color, which could be clearly differentiated from the greenish trace produced by negative controls (healthy leaves) that is due to the presence of chlorophyll. Moreover, the use of the adsorbed antibody with the diluted healthy leaf extract reduced chlorophyll color development, resulting in the enhancement of the specificity of R-BIA for detecting the two pepper viruses.

The sensitivity and specificity of R-BIA in detecting PMMoV and PepMoV were tested. For the sensitivity test, purified PMMoV (200 μg/ml) [5] was mixed with an equal volume of healthy pepper leaf sap (0.05 g/ml), and 10-fold serial dilutions were made in 0.1 M Tris-HCl (pH 7.4). These were subjected to R-BIA as described above, using the optimal reaction conditions of TBIA shown in Table 1. In this test, the R-BIA could detect as little as 1 ng/ml of virus without nonspecific reaction (Fig. 2A). R-BIA also showed a high sensitivity, similar to dot-blot imunobinding assay (data not shown). This level of sensitivity achieved using R-BIA is as high as that of ELISA [20], DBIA [7], and tissue-print hybridization [2], which are all sensitive enough to detect 1 ng/ml of viral antigen.

To verify the specificity of the R-BIA, the ring blot of PepMoV-infected pepper leaves was compared with RT-PCR. For RT-PCR, viral RNA was isolated from two leaf discs from the same leaves used for R-BIA, as described by Singh in 1998 [17], with the final product eluted in 50 μl of diethylpyrocarbonate-treated water. Reverse transcription was performed using oligo-dT [18] for PepMoV in a 20-μl volume, following the manufacturer's protocol (Promega, Southampton, U.K.). PCR was carried

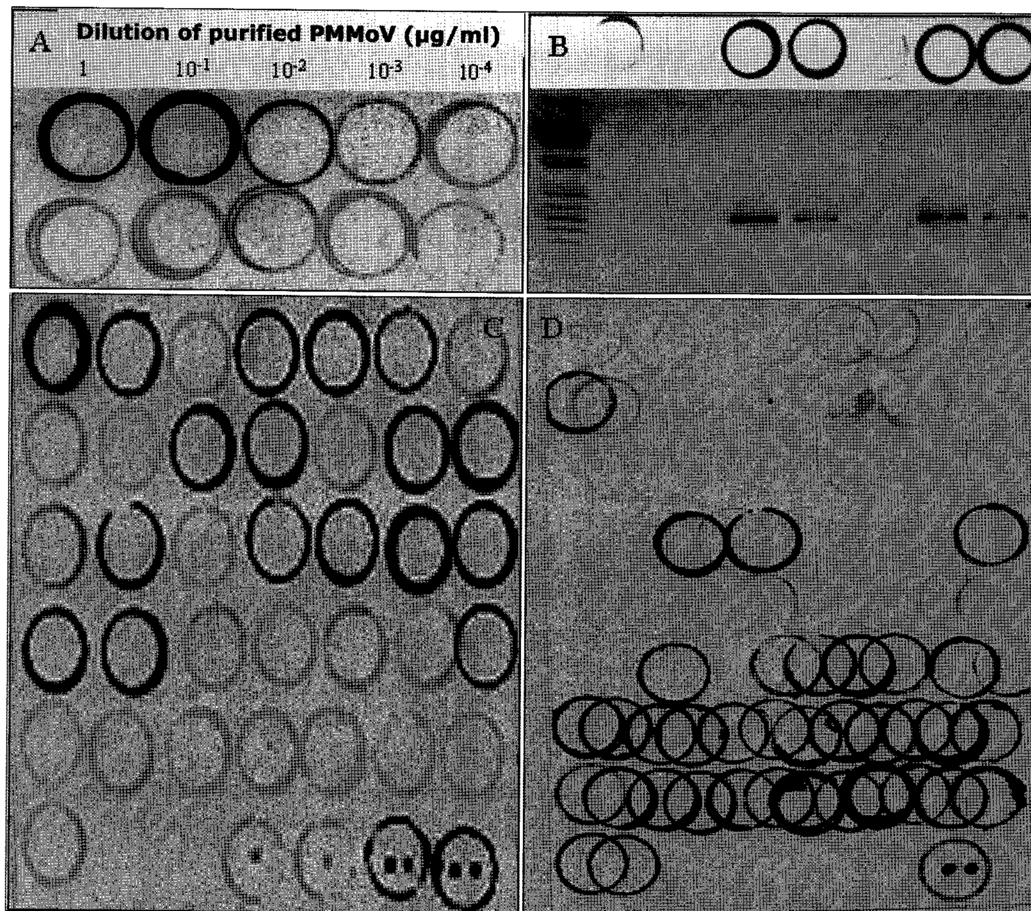

Fig. 2. Ring-blot immunobinding assay (R-BIA) showing sensitivity (**A**), specificity (**B**), and utility (**C**, **D**) for the detection of pepper mild mottle tobamovirus (PMMoV) (**A**, **C**) and pepper mottle potyvirus (PepMoV) (**B**, **D**) in pepper leaves.

Ring-blots of two- and ten-fold serial dilutions of leaf sap mixed with purified PMMoV (200 μg/ml) were obtained by pressing the larger open side of commercially available micropipette tips wetted with each dilution onto a nitrocellulose membrane (**A**). R-BIA and RT-PCR were carried out with ring-blots and RNA templates from the same leaf (**B**). Representative results of PMMoV (**C**) and PepMoV (**D**) detection by R-BIA, which was performed as described in Table 1. Healthy leaf samples developed faint green or brownish-purple colors, whereas diseased leaf samples developed a dark purple color. Ring-blots of negative and positive controls are marked by one and two dots in the center of the circle, respectively.

out using 3 μl of cDNA dilution (1:5) in a 20-μl PCR mix containing 2 μl of 10×PCR buffer (Dakara, Dakara Korea Biomedical Inc., Korea), 0.1 μl of 10 mM dNTP mixture (Dakara), 0.2 μl of 5 U/μl *Taq* polymerase (Roche, Indianapolis, IN, U.S.A.), and 2 μl of each primer (10 pmol/l). Primer sequences were designed using the Primer 3 software (Genetics Computer Group Inc., Madison, WI, U.S.A.). The sequences were 5'-TGGGTCTGGCTCGATACGCA-TTTGA-3' (sense for PepMoV), and 5'-CTCGAGTTTTT-TTTTTTTTTTTT-3' (antisense for PepMoV). The PCR was carried out on a Peltier PCR machine (PTC-200, MJ Research, Watertown, MA, U.S.A.) using the following parameters: denaturation for 3 min at 90°C, 35 cycles of denaturation for 1 min at 90°C, annealing for 1 min at 57°C, and primer extension for 1 min at 72°C, followed by one final extension for 5 min at 72°C. PCR products were analyzed by electrophoresis through a 0.8% agarose gel followed by staining in ethidium bromide and visualization of DNA bands using a UV transilluminator. The results of R-BIA were in accordance with the RT-PCR results, suggesting that R-BIA has high specificity for PepMoV detection (Fig. 2B).

To use R-BIA for medium-sized screening, experiments using the pepper viruses were conducted. PMMoV samples were collected from commercial chili peppers grown in the greenhouses at the experimental farm of Seoul National University, Suwon, Korea. PepMoV samples were prepared by infecting F_2 progenies from the cross of Chilsungcho (susceptible to PepMoV) and *Capsicum annuum* CM334 (resistant to PepMoV) with PepMoV-SNU1. A total of 2,200 samples, consisting of 2,000 for PepMoV and 200 for PMMoV, were screened for each virus infection. These samples were tested with R-BIA in conjunction with healthy (negative) and diseased (positive) controls, which are indicated by one or two dots inside the ring-blot, respectively (Figs. 2C and 2D). The majority of positive reactions produced complete continuous circles, and a few

produced broken circles that could be clearly differentiated from the negative reaction circles by comparing color and density. The leaf sap did not overflow to adjacent overlapping circles during blotting, so no cross-contamination occurred, which could have given rise to confusion in discriminating reactions between infected and non-infected samples.

R-BIA requires a small amount of membrane for sample blotting, which allows more samples to be deposited on a membrane, compared with when DBIA and TBIA are used [1, 8, 18, 22]. Therefore, considering this and the other aspects presented in our study, R-BIA provides a simple and rapid method for virus detection that requires only 4–5 h for the entire process. This method provides a very useful tool to perform preliminary screening for PMMoV or PepMoV infection in pepper samples on a medium scale, because it is sensitive, specific, economic, and easy to perform. It may also be a reliable method for the detection of other plant viruses on a medium to large scale, using minimum equipment such as plastic containers and a shaker. R-BIA could be broadly used for the rapid and simple screening of virus infection in laboratories without the need for elaborate equipment for virus detection.

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