African Journal of Biotechnology Vol. 8 (5), pp. 751-759, 6 March, 2009
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2009 Academic Journals

Full Length Research Paper

Development of rapid, specific and sensitive detection of *Cucumber mosaic* virus

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Accepted 23 November, 2007

Cucumber mosaic **virus (CMV) causes major losses to thousand agricultural and horticultural crops around the world. Unlike other plant pathogens, there are no direct methods available yet to control viruses and, consequently, the current measures rely on indirect tactics to manage the viral diseases. Hence, methods for detection and identification of viruses, both in plants and vectors, play a critical role in virus disease management. A rapid assay for diagnosis of CMV, which can be employed in both laboratory and field, is essential. Therefore, this study was undertaken to develop a procedure for detection of the CMV in infected plants using a monoclonal and polyclonal antibodies. Dot-immunobinding assays (DIBA) are useful alternatives to microtitre plate enzyme-linked immunosorbent assay (ELISA). Nine monoclonal antibodies were readily used for detected CMV by TAS-ELISA and DIBA of infected plants. DIBA has about the same sensitivity as ELISA in microtiter plates, but it has the additional advantages of simplicity, quick completion in the field or office on large numbers of samples, economy, and can be quantified using densitometry.**

Key words: Monoclonal antibodies, polyclonal antibodies, triple-antibody sandwich, enzyme-linked immunosorbent assay, dot-immunobinding assays.

INTRODUCTION

Cucumber mosaic virus (CMV) is a type of species of the genus *Cucumovirus,* belongs to family, *Bromoviridae*, and is a tripartite (+) single stranded RNA virus. It is distributed worldwide and has a very large host range, infecting more than 1000 plant species (Roossinck, 2002). It is aphid transmissible in a non-persistent manner by more than 60 aphid species, and causes severe diseases in various crops all over the world. The CMV genome has been extensively studied, and replicase functions have been attributed to RNAs 1 and 2 encoded proteins 1a and 2a, whereas RNA 3 encodes the viral movement protein 3a as well as the viral coat protein in a subgenomic manner (Palukaitis et al., 1992). In addition RNA 2 has a second overlapping open reading frame encoding protein 2b (Ding et al., 1994). *C. mosaic* virus strains are separated into two subgroups- I and II, which differ in their nucleotide sequence and can be distinguished by polymerase chain reaction (Rizos et al., 1992).

Plant diseases caused by pathogens are of great economic importance. Damage to crop plants due to virus's infections is difficult to assess and actual figures for global crop loss are not available. Plant diseases losses are estimated to be $60 billion annually (Hsu, 2002). Unfortunately, there are also no economically feasible chemical agents similar to fungicides and bactericides that are effective against plant viruses. Strategies aimed at plant virus disease management are largely directed at preventing virus infection by: (i) eradicating the source of infection to prevent the virus from reaching the crop, (ii) minimizing the spread of the disease by controlling its vector, (iii) utilizing virus-free planting material, and (iv) incorporating host-plant resistance to the virus. An essential precursor of the implementation of control measures,

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however, is an accurate diagnosis of a virus disease and mapping of its geographical and temporal distribution in an area or crop. Because of the increased worldwide movement of germplasm through seed and other propagative material in global trade and agriculture, diagnosis of viruses in these materials assumes greater importance for national quarantine services to ensure the safe movement of germplasm across the borders.

Many methods have been developed for the detection and identification of plant viruses. A single diagnostic test or assay may provide adequate information on the identity of methods for the detection of plant virus diseases, but a combination of methods is generally needed for unequivocal diagnosis. Optimally, methods for detection of plant viruses are sensitive, specific, and can be completed within a relatively short period of time and are inexpensive. Recent advances in techniques for the detection of proteins and nucleic acids have provided an opportunity to develop methods with these qualities for the diagnosis of plant virus diseases. The type of diagnostic test used ultimately depends on resources, facilities, availability of reagents, level of specificity and sensitivity required expertise and skills available to carry out these assays, type and number of samples to be tested, and the amount of information available on the virus to be detected. The most important criteria in evaluating diagnostic techniques are reliability, sensitivity, and cost. The direct double antibody sandwich (DAS) microtitre plate format of ELISA first introduced to plant virology by (Clark and Adams, 1977) is still widely used. This assay is very sensitive, detecting viruses at concentrations of 1-10 ng/ml (Torrance and Dolby, 1984). ELISA has been the method of choice for the detection and assay of various plant viruses because it is sufficiently sensitive for most applications. However, there are occasions when it may not be sufficiently sensitive, e.g. detection of very low concentrations of virus present in vector insects (Torrance, 1987), or potato virus Y-infected dormant potato tubers produced on plants infected during the growing season. Also polyclonal antibody-based DAS-ELISA may fail to detect serologically related strains because of the rather narrow strain specificity it exhibits (Koenig, 1981). Therefore, other more sensitive ELISA methods may be needed for their detection.

Various alternative ELISA formats and substrates have been published over the years which claim to improve the limit of detection and broaden strain specificity (Cooper and Edwards, 1986). Incorporation of monoclonal antibodies has provided improvements, for example, by decreasing non-specific background reactions and providing more accurate discrimination between strains of barley yellow dwarf virus (Torrance et al., 1986). The biotin–avidin modification (Strasburger and Kohen, 1990) in which the high affinity of avidin for biotin is exploited has proved versatile and sensitive. Also, it was shown that biotin-labelled antibodies specific for tobacco mosaic virus detected serologically related tobamoviruses, thus broadening the range of serologically related virus isolates detectable by DAS-ELISA (Zrein et al., 1986). Nowadays, it is more usual to use streptavidin (from *Streptomyces avidinii*) to decrease possible problems of non-specific binding of avidin (Strasburger and Kohen, 1990). In these assays the primary (virus-specific) antibodies are biotinylated, and biotin bound antibodies revealed by reaction with a universal streptavidin-enzyme conjugate. Recently, an assay which incorporates biotinylated antibodies, and conjugates comprising streptavidin coupled to homopolymers of HRP improved detection sensitivity by 12 – 25 times over a monoclonal antibody-based DAS-ELISA in assays to detect barley stripe mosaic virus.

Dot-immunobinding assays are valuable because they are simple, sensitive and rapid. It is widely used in the field of clinical chemistry and cell biological studies. The protein of interest is detected by reaction with a primary antibody followed by a labelled second antibody. The second antibody is conventionally labelled by enzyme. Consequently, it is a potentially useful research and diagnostic tools in plant pathology, particularly in the study of pathogens transmitted by vectors. The low volume of the sample required for dot-immunobinding assays is an important advantage over ELISA in analyzing samples without dilution. Recently Sulimenko and Dráber (2004) have reported that dot-immunobinding assay is a simple immunoassay method to detect and quantify immunoglobulins proteins in hybridoma culture supernatant.

The purpose of this study was to increase the sensitivity of ELISA for CMV diagnosis by modifying the test, in particular with the production and use of a monoclonal antibody in a triple antibody sandwich (TAS)-ELISA and to determine the sensitivity and specificity of the dot-immunobinding assay, to compare its validity, and quantification of CMV in infected plant sap.

MATERIALS AND METHODS

Virus purification and monoclonal and polyclonal antibodies production

Pepo-CMV and another tested isolates were kindly provided by Dr. Satoshi Ohki, Plant Pathology Department, Graduate School of Agriculture and Biological Sciences, Osaka Prefecture University, Osaka, Japan. Pepo CMV was isolated in greenhouse from 50 g of infected tobacco, *Nicotiana tabacum* cv. Xanthi-nc leaf tissue. The sample was homogenised in a blender with 1:1 (w/v) extraction buffer (0.5 M sodium citrate pH 6.5, 5 mM EDTA and 0.5% thioglycerol). The homogenate was expressed through muslin, emulsified with one vol chloroform and centrifuge at 15 000 × *g* for 10 min. The aqueous layer was removed and the virus precipitated with 10% polyethylene glycol (Mol. Wt. 6,000) stir for 30 - 40 min at 4˚C. After Centrifugation as before the pellet was resuspend in 5 mM tetraborate pH 9.0 with 0.5mM EDTA, 2%Triton X-100 and the centrifuge step repeated. Virus was pelleted by centrifugation of the supernatant at 360,000 × *g* for 45 min and resuspend in 5 mM tetraborate pH 9.0 with 0.5 mM EDTA and 2% Triton X-100. This suspension was applied to sucrose gradient and after centrifugation at 140,000 × *g* for 2 h the virus bands were removed and diluted

fourfold with 5 mM sodium tetraborate pH 9.0 with 0.5 mM EDTA. Virus particles were pelleted by centrifugation at 360,000 × *g* for 45 min and resuspended sodium tetraborate pH 9.0 with 0.5 mM EDTA. Virus concentration was estimated by measuring absorbance at 260 nm and using as extension coefficient of 5.0.

Immunized eight-weeks old BALB/c mice were injected subcutaneously with 100 µg in a volume 0.1 ml phosphate-buffered saline (PBS; 0.01M phosphate and 0.015 M sodium chloride, pH 7.5) of purified CMV of either strain Pepo or M2, which were mixed with an equal volume of adjuvant containing TDM plus MPL. After three injections were administered at two-week intervals, three days after the fourth injection, the mice were given a peritoneal injection of 200 µg of virus in 0.1 ml PBS. The mice were sacrificed 3 days later and their spleens were harvested. Fusion experiments were carried out in which lymphocytes from the spleens of the immunized mice were mixed at a 5:1 ratio with non-secreting P3X63-Ag8-U1 myeloma cells in Polyethylene glycol 6000 at 50% (w/v). The cells were distributed to 96 well plates at a concentration of 10^5 cells/well with medium HAT (100 µM hypoxanthine, 0.4 µM aminoprotein, 16 µM thymidine, 6 mM Hepes, and 200 µM 2-mercaptoethanol).

Clones, which successfully secreted antibodies specific to CMV, were examined with both ELISA and western blotting. Positive clones were additionally subcloned by limiting dilution method in the presence of thymocytes of BALB/c mice as feeder cells as follows: the cells of each well were counted in a Burker chamber to reach a concentration of 1 cell per 200 µl of medium in the well. After 3 - 4 days, clones were screened by ELISA on the plates coated with CMV-CP, and the cells from the positive wells were used to repeat the cloning procedure twice more. Monoclonality of the cells in the wells was evaluated by colony morphology, and then the clones were cultured in a 5 ml volume and stored in liquid nitrogen. Since 1999, the above hybridoma clones have been used many times for the production of antibodies using repeated cultures. The isotype of the MAbs was determined with a mouse MAb isotyping kit (Amersham) following the manufacturer's instructions.

The production and purification of the monoclonal antibodies specific CMV-CP

The frozen hybridomas were retrieved from liquid nitrogen, rapidly thawed with constant agitation, and cultured in DMEM medium (Sigma, St. Louis, MO) containing 15% FCS (10^6/ml of medium). Two female mice (BALB/c, 10 weeks old) were treated with 0.5 ml of pristine. 10 days prior to inoculation, the mice were injected intraperitoneally with 10^6 hybridoma cells. The hybridomas grew as ascites tumors in peritoneal space, and ascitic fluids containing antibodies were collected 10~15 days after the inoculation. The collected ascitic fluids were pooled, and the fluids or corresponding IgG fractions were stored at 4°C until further use. The immunoglobulin fraction was separated from ascitic fluid by using protein-A affinity purification column (Bio-Rad, Hercules, CA). Ascitic fluid was diluted with binding buffer 1:2 (v/v), centrifuged, and filtered through a PF syringe filter, 0.2 µm Acrodisc. Nine milliliters of the diluted ascitic fluid was applied to a column filled with 3 ml of sorbent and then allowed to flow through the chromatographic column with immobilized protein-A. The column was washed with 10 bed volumes of washing buffer, eluted with 3 - 4 bed volumes of eluting buffer, and neutralized with Tris-HCl buffer. After dialysis against PBS buffer, the IgG was incorporated into indirect ELISA to establish assay parameters of the respective antibodies.

Production of rabbit polyclonal antibodies, using Pepo-CMV immunized rabbit, after various injections, an antiserum was obtained which showed a titre 1/10,000 against the homologous isolate with ELISA and no detectable reaction with healthy samples after absorption with healthy plant preparations. Polyclonal antibodies were purified from antiserum by precipitation with ammonium sulphate and passage through a column protein A as mentioned above.

Biotinylation of MAbs

The purified MAbs were dialyzed against 0.02 M sodium carbonate buffer (pH 9.5) and then diluted to 1 mg/ml. Biotinamidocaproate N-hydroxy succinimide ester (NHS biotin, Sigma) was dissolved in distilled water to 1 mg/ml. The NHS-biotin was added (0.1 ml per 1 mg of antibody) to the MAbs solution and the mixtures were gently stirred for 1 h at room temperature. The reaction products were dialyzed against PBS to remove any non-reacted NHS-biotin. BSA was added to the biotin-labelled antibody to 1% as a stabilizer.

Indirect antigen coat plates ELISA procedure

One hundred microliters of CMV (1µg/ml) in the coating buffer was added to each well of a 96-well microplate (Iwaki, Funahashi, Japan). The plate was incubated overnight at 4°C. After the buffer was removed, the wells were washed three times with 200 µl of PBST. One hundred microliters of standard in the assay buffer and 50 µl of ascitic fluid (or IgG) diluted in PBS-0.05 % Tween (PBST) were added into the wells. After 1 h of incubation at 37°C, the unbound compounds were removed by washing (five times) with assay buffer. One hundred microliters of goat anti-mouse IgG (H+L) specific, conjugated with horseradish peroxidase HRP (Amersham Pharmacia Biotech), plates were incubated at 37°C for 1 h. After the solution was removed, each well was washed four times with the washing solution. The absorbance of the developed color was measured at 450 nm.

TAS-ELISA

Assay conditions were optimized by checkerboard titration of rabbit polyclonal antibodies standard. Microtitre plates were coated with 100 µl of PAbs diluted 1:5000 in 0.05 M bicarbonate buffer (pH 9.6) at 4°C overnight, followed by 60 min incubation at 37°C. After repeated washing, the plate with 0.15 M phosphate-buffered saline (PBST) non-specific binding sites was blocked by adding 1% Block Ace in PBS at 37°C for 1 h. Then, the plate was washed five times with PBST. Following the last wash, CMV-CP samples were diluted in PBS buffer incubated at 37°C. Followed by MAbs secondary antibody dilution with their optimum concentration incubated at 37°C for 1 h. Then, the plate was washed five times with PBST. 100 µl of goat anti-mouse (H+L)-specific HRP-conjugated (Sigma Chemical) third antibody solution (1:5000) in TBST was incubated in each well for 1 h at 37°C.

Dot-immunobinding assay

Young upper leaves of infected and non-infected plants were rolled longitudinally into a tight scroll. The scroll was folded once before the leaf tissue was cut with a razor blade. The cut leaf surface was immediately held on the surface of a PVDF membrane for 6 - 8 s and/or tissues were homogenized with extraction buffer, purified CMV diluted with healthy plant sap 10^{-2} gently drooping 2 µl onto membranes of PVDF of 0.45-µm pore size (Bio-Rad). The membrane was air-dried and blocked for 30 min in TBS buffer (10 mM Tris-HCl, pH 7.4, 0.15 M NaCl) containing 50 g L^{-1} defatted milk powder (TBS-milk buffer) or in PBS buffer (8 mM Na_2HPO_4 1.5 mM KH_2PO_4, 2.7 mM KCl, 3 mM NaN_3, pH 7.4) containing 20 g L^{-1} Triton X-100 and 50 g L^{-1} defatted milk powder (PBS-milk buffer). In the latter case, the membranes were washed three times in distilled water. Membranes were incubated for 60 min in 1µg/ml MAbs diluted with TBS-milk buffer or PBS-milk buffer. After three washes

Table 1. *Cucumber mosaic virus* immunoglobulin class and subclasses of monoclonal antibodies, and the cross reactivity to CMV subgroups.

Fusion Subgroup specificity[a]	Clones	Immunogen	Subclass	Optimum		
				µg/ml	I	II
1	4	Pepo	IgG1	0.20	+ +	-
	5			0.05	+ + +	-
	6			0.15	+ + +	-
2	8	Pepo	IgG1	0.45	+ +	-
3	7	Pepo	IgG2b	0.80	+	-
4	M2-1	M2	IgG1	2.0	-	+ +
	M2-2			2.0	-	+ +
5	M2-3	M2 + Pepo	IgG1	5.0	-	+
	M2-4		IgG1	10.0	+ +	+ +

[a] The specific reactivity of the MAbs specific binding activity against CMV-CP. The prescreening of the MAbs with enzyme linked immunosorbent assay were scored as (+ or -) for measurements corresponding to absorbance (450 nm) values < 0.5 (-), 0.5-1 (+), 1-1.5 (++), 1.5 -2 (+++).

of 5 min each with TBST or PBST (TBS or PBS plus 3 g L^{-1}Tween 20) and two washes, the first and the last with distilled water, membranes were incubated for 60 min in a 1/5000 dilution in TBS-milk or PBS-milk buffer with appropriate goat anti-mouse alkaline phosphates AP-conjugated antibody (Amersham Pharmacia Biotech). Membranes were then washed as before and equilibrated in substrate buffer (0.1 M Tris-HCl, pH 9.5) for 5 min before adding the substrate. All incubations were at room temperature. AP activity was detected with the chromogenic substrate (5-bromo-4-chloro-3-indolyl-phosphate/4-nitroblue tetrazolium chloride, BCIP/NBT).

Quantification of CMV with DIBA

Purified Pepo-CMV was diluted with healthy plant sap and incubated with membrane, consequently probed with MAb-5 as written above. The visualized spots were scanned and quantified, using a 12-bit transparency scanner (Image Scanner, Amersham Biosciences). Changes in spot intensity were analyzed by Image Master two-dimensional Elite software (Amersham Biosciences) on 12-bit TIFF images using background subtraction by the lowest-on-boundary method. As the absorbance density of the spots was related to the amount of loaded CMV, calibration curves from standards were prepared by plotting the measured absorbance against the amount of CMV. Concentrations of CMV in the tested sample were then determined by comparing the corresponding absorbance with the calibration curve.

Western blot analysis

One hundred milligram of fresh leaf material was homogenized in SDS extraction buffer according to Laemmli, (1970). Samples were boiled for 3 min and then centrifuged for 5 min at 10,000 × *g*. Ten microliters of the supernatant were subjected to SDS gel electrophoresis in 10% polyacrylamide gel, consequently, proteins were blotted onto PVDF membrane by electroblotting. Membranes were blocked as mentioned above overnight and then probed with MAb-M2-4 alkaline phosphatase-labelled goat anti-mouse IgG (PIERCE), hence the bands were visualized with incubated substrate as mentioned above. All other chemicals were purchased from (Nacalai Tesque, Inc. Kyoto, Japan).

RESULTS

The antibodies bind a broad variety of antigens with high affinity and specificity and the structural information about the molecular interactions between the antibodies and antigens helps us to understand the effect of mutations on the affinity and specificity of the antibody. Monoclonal antibodies (MAbs) were prepared against *C. mosaic* virus (CMV) coat protein Japanese M2 and Pepo strains. MAbs were characterized and used to develop a diagnostic with high sensitivity and specificity against virus coat protein. Fusion of splenocytes of immune mice with X63Ag8.653 myeloma resulted in a number of hybridomas, allowed the production of hybrid cell lines from B cells which secreted a single, monoclonal antibody with one binding site specificity. Furthermore it can potentially produce unlimited quantities. However, the serological differentiation of CMV isolates is of importance in breeding for disease resistance and in studying disease epidemiology. So specific monoclonal antibodies have been developed and one of the goals in their development was to provide tools for serotyping isolates of the virus.

Nine clones were selected for the study because their anti-CMV activity was detected by solid phase ELISA and western blotting, when the culture supernatants were diluted 100-1000-fold. Antibodies of these clones (after two reclonings) were purified from ascitic fluid by affinity chromatography (Figure 1). Antibodies of each clone were conjugated with biotin. The data of Table 1 indicate that the selected antibodies bind to three different epitopes of CMV. MAbs- (4, 5, 6, 7, 8, and 10) were bound specifically to Pepo-CMV might same epitope (or adjacent epitopes); no cross reactivity with other strain. MAbs- M2- (1, 2, and 3) were bound specifically to M2-CMV. On the contrary, M2-4 antibody showed cross reactivity with both strains M2- and Pepo-CMV. Conse-

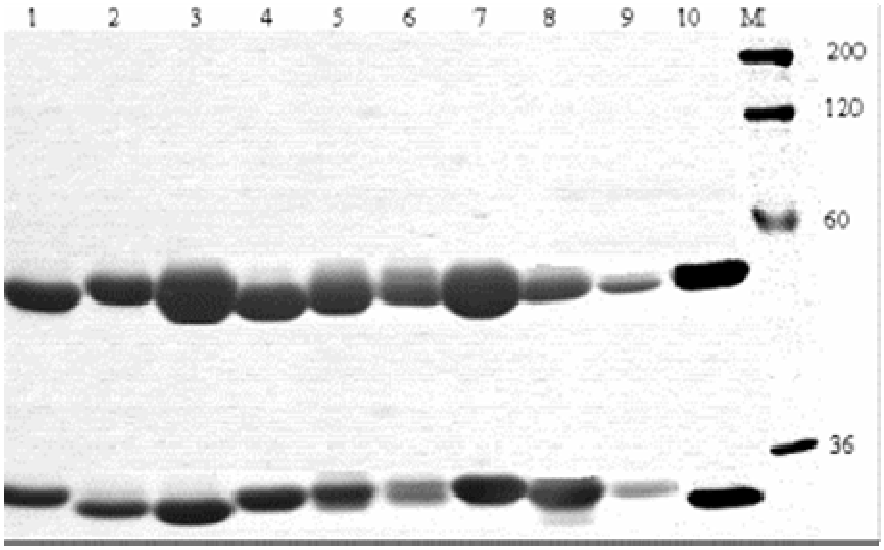

Figure 1. The purification of the monoclonal antibodies specific CMV coat protein with affinity protein A column. Electrophoresis of purified mouse monoclonal immunoglobulins IgG were separated on 10% SDS-PAGE under reducing condition (2-mercaptoethanol) followed by stained with Coomassie Brilliant Blue R 250. Where, MAbs- (4, 5, 6, 7, and 8) numbering 1 to 5, while MAbs- M2 (1, 2, 3 and 4) numbering 6 to 9, while PAbs-IgG1 number 10. Molecular marker in kDa is indicated on the right.

quently, this MAb showed high reliability to detect various strains and isolates of native CMV particles using an immunobinding-dot binding as well as CMV coat protein denatured by boiling in SDS and 2-mercaptoethanol. Therefore, the MAbs-M2-4 has a capability to detect an epitope of all strains and isolates of CMV coat protein (Figure 2). However, the reactivity of recognizing sharing epitope which is present in both I and II subgroup, suggests that this epitope is highly conserved among these CMV strains. Consequently, Antibody-based resistance is a novel strategy for generating transgenic plants resistant to pathogens. The potential of recombinant antibodies to interfere with the infection of a plant virus was demonstrated in 1993 (Tavladoraki et al., 1993). Hence, our prospect for long term goal is that the recombinant MAb-M2-4 gene potentially protects a wide range CMV strains.

Development of TAS-ELISA for detection of CMV-CP

Standard solid-phase ELISA with monoclonal antibodies involves the formation of a complex between antibody adsorbed on a solid phase (capture antibody), antigen, and antibody to another epitope of the antigen, antibody conjugated with an enzyme. Thus, a sandwich is formed: solid phase capture antibody-antigen-antibody. We have studied the influence of adsorption on a solid phase of polyclonal antibodies to different epitopes of CMV. The sensitivities of the TAS-ELISA for detection of CMV were different between MAbs specific strains, and several of MAbs were tested for their ability to function either as coating antibody on the solid phase or as a biotin conjugate. It is known that not all antibodies are equally suited as reagents in the different steps of ELISA (Porta et al., 1989), since certain MAbs may loose some of their activity after absorption to plastic or after labeling (Dekker et al., 1989). The specific reactivity of different MAbs revealed that it is possible to detect as little as 10 pg per well (10 ng/ml) of purified virus for Pepo-CMV, while as little as 1 ng per well (1 µg/ml) for M2-CMV (Figure 3). Most of CMV strains had weak immunogens. Nevertheless, in the present study of the M2-CMV strain, the amount of accumulated virus in an infected tobacco plant was so low that it required about a month to yield 2 mg from 100 g of infected leaves. Accordingly, it had very weak immunogens probably due to its instability, and yield low-tittered antisera, with uncomplimentary binding affinity of their MAbs (Palukaitis et al., 1992). On the contrary, Pepo-CMV had very fast accumulation rate requiring only about 5 days, accumulated a high virus yield of 1 mg from 1 g of leaves and had a powerful immunogen high titre antisera with very high binding affinity of their MAbs (data not shown).

Development of the detection CMV-CP DIBA

Nitrocellulose or PVDF membranes filters have been widely used in molecular biology to immobilize nucleic acids. In recent years, it has been found that this material also binds proteins with high efficacy. The immobilized

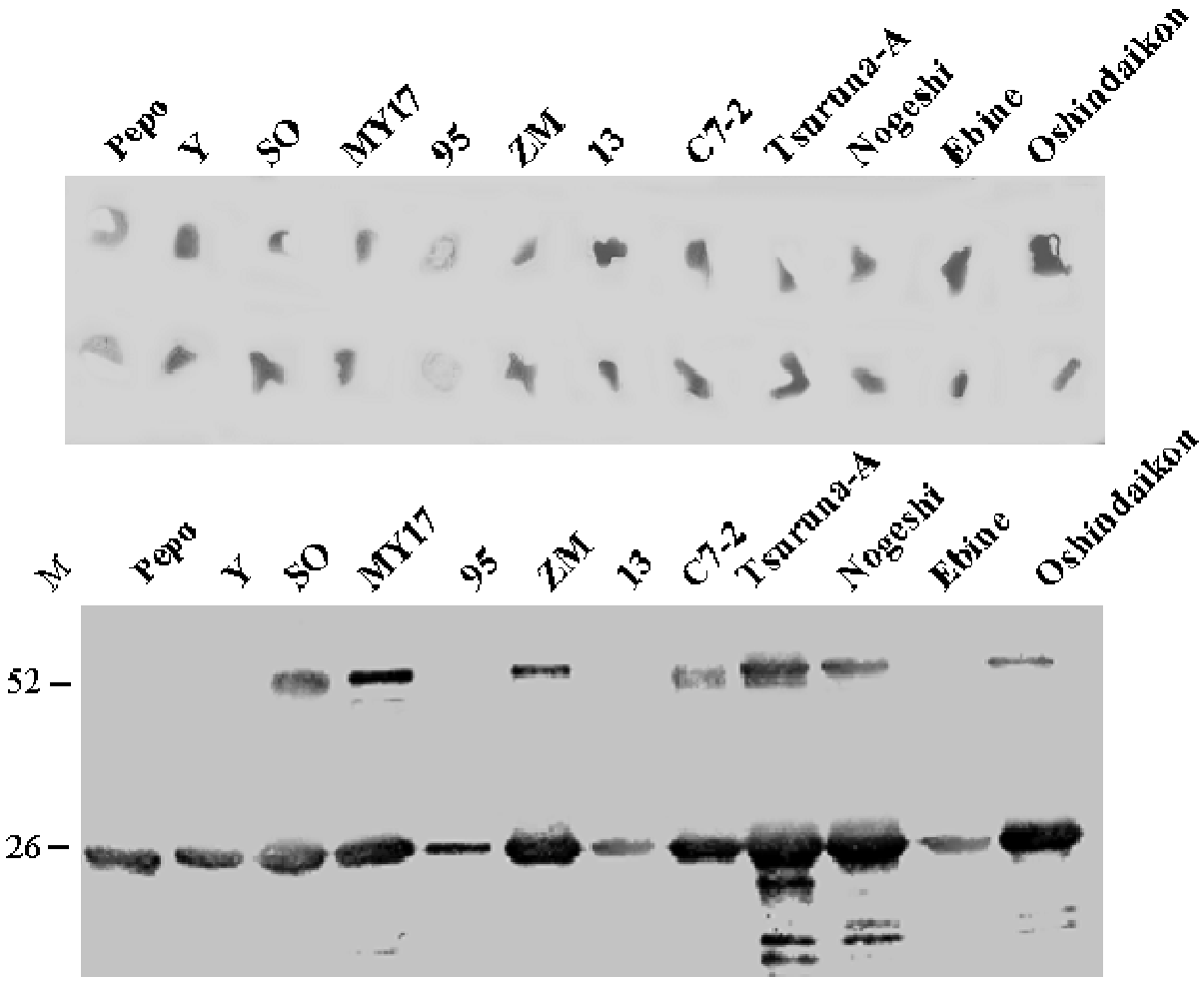

Figure 2. Immunobinding assay for detection survey CMV stains and isolates. (A) Dot-immunobinding assay for detection infected tobacco plants infected with CMV strain as shown in the legend, direct blotting of infected leaf to PVDF membrane. (B) Western blotting analysis, same strains and isolates of CMV infected plants. The membranes containing proteins were probed with MAb-M2-4 (10 µg/ml), which showed cross reacted with all tested of CMV isolates showing 26 kDa band of coat protein and its 52 kDa dimer; in native and denaturized form of CMV coat protein. Molecular weight marker (kDa) indicated in the left side.

proteins can be analyzed by subsequent binding of specific antibodies and appropriate detection molecules. A main application has been the electrophoretic transfer of proteins from gel electropherograms to nitrocellulose membranes ("immunoblotting"). Direct spotting of small amounts of antigens has also been used for screening of antibodies reactivity and for determination of antibody specificity. However, there appears recreantly the use of these membrane filters as the basis for a quantitative assay of bound immunoglobulins in hybridoma culture supernatants (Sulimenko and Dráber, 2004) by an antibody-based detection system.

The sensitivities of MAbs were compared for detection of CMV. CMV could be readily detected both in purified virus preparations and in crude sap of infected tobacco leaves by DIBA on PVDF membrane (Figure 4). The minimum detection levels were 128 ng/ml with purified preparation of Pepo-CMV (Figure 4-A) and at 10^{-5} dilution of extracts from infected leaves (Figure 4B). In agreement with ELISA results, the detectability of M2-CMV, when tested with DIBA as well, showed lower detectability than Pepo-CMV. The minimum detection level of the purified M2-CMV was 3.2 µg/ml (Figure 4A), while infected tobacco leaves with M2-CMV can be detected at dilution limit of 10^{-3} (Figure 4-B). The sensitivity of the different antibodies was compared for detecting the virus in infected tobacco plants. The results obtained with limiting dilution of infected plant with Pepo-CMV, MAbs- (4 and 5) showed the highest specific reactivity limited detection at 10^{-4} dilution. MAbs-(6, 7, and 8) showed intermediate limit detection at 10^{-3} dilution, while MAb-10 revealed lowest detection limit at 10^{-2} dilution (Figure 4B). M2-CMV specific MAbs showed a lower delectability with limiting dilution range 10^{-1}~10^{-3} compa-

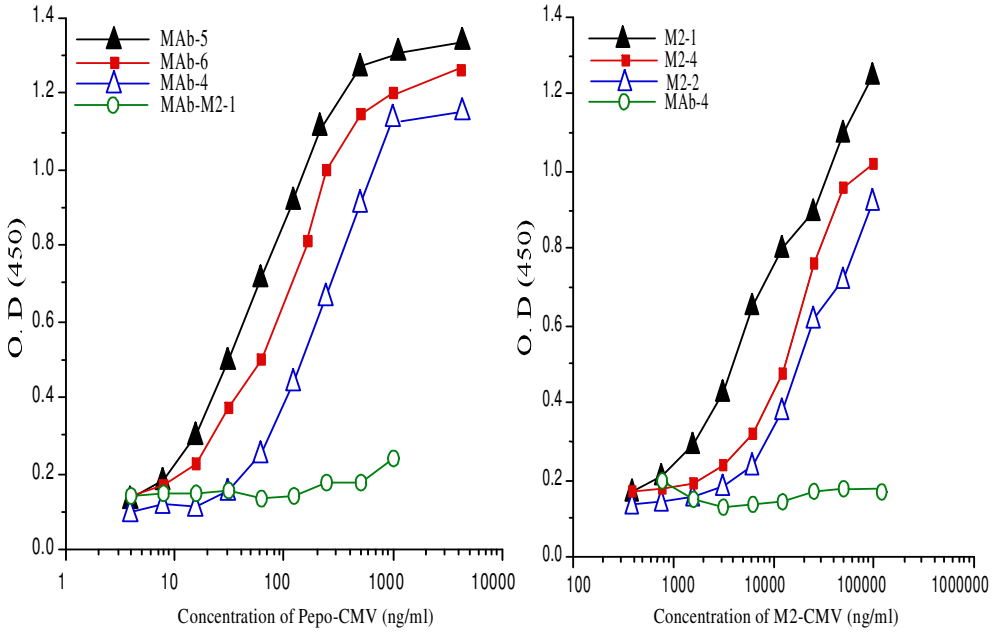

Figure 3. TAS-ELISA for detection of the purified Pepo-, M2-CMV. Plates were coated with polyclonal antibodies (dilution: 1/5000) first layer for trapping CMV coat protein. The antigens were used at serial concentration (0.001~10 µg/ml) with Pepo-CMV (A) which detected with MAbs- (4, 6, and 8) which are specific CMV subgroup-I. While M2-CMV was used at concentration (0.1~100 µg/ml) detected with MAbs- (M2-1, M2-2, and M2-4) specific CMV subgroup-II. The anti-mouse (H+L) IgG-HRP was diluted at 1/3000.

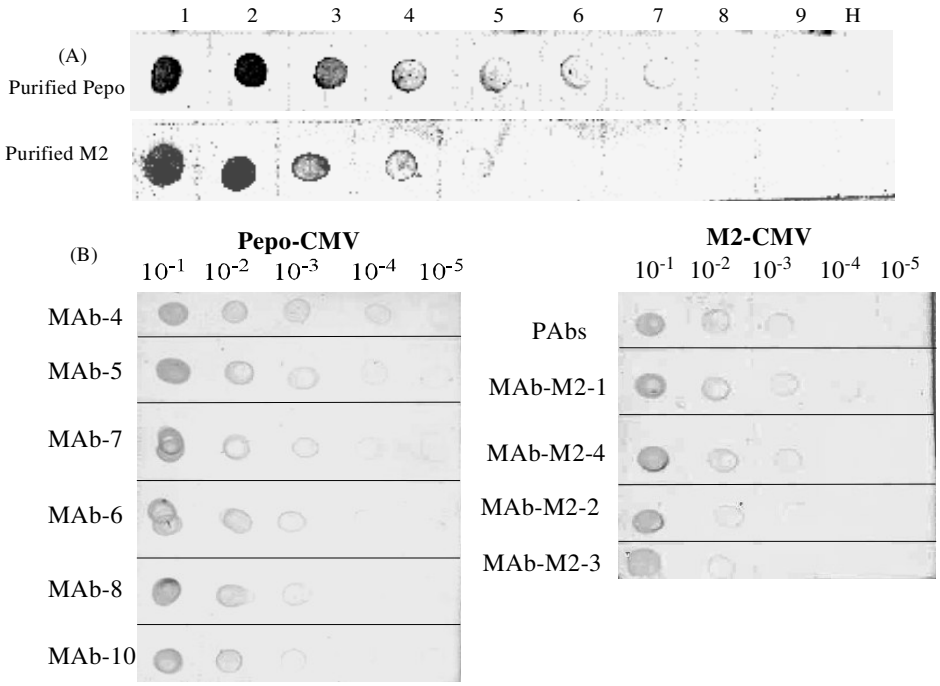

Figure 4. Dot-immunobinding assay detection of dilution endpoint of 2-µl aliquots of the detection limit of CMV coat protein. (A) Purified virus preparations Pepo-CMV strain of 2 µg (spot), and serial five times dilution in each next spots; the limiting detection 128 pg (spot 7), while limiting detection of purified M2-CMV 3.2 ng (spot 5). (B) Detection of CMV in leaf extracts serial dilution 10^{-1} up to 10^{-5}. Detection limit of MAb-4 with Pepo-CMV at 10^{-5}, while M2-CMV is at 10^{-3}.

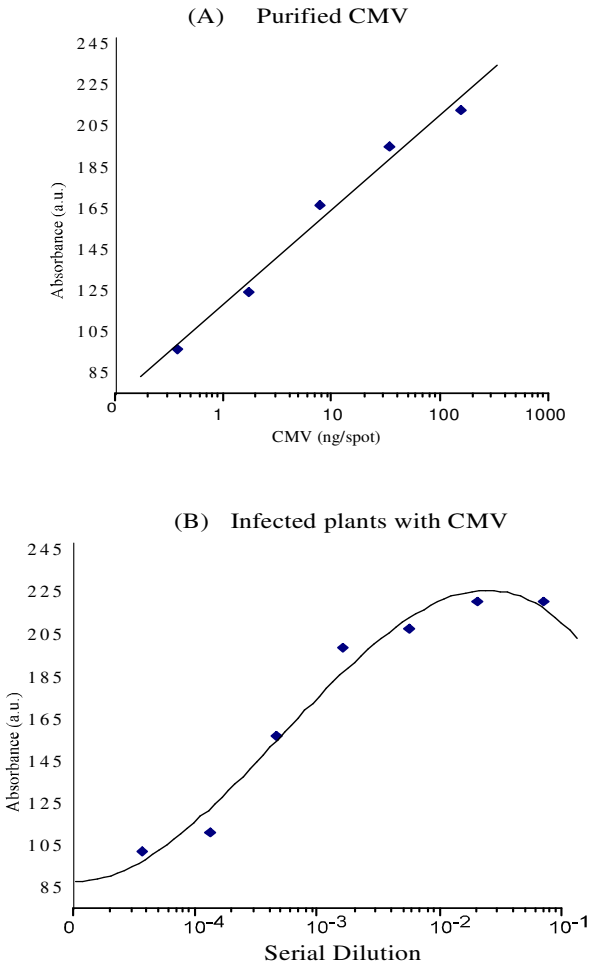

Figure 5. Relationship between absorbance (arbitrary units, a.u.) of immunostained spots and the amount of loaded purified CMV diluted with healthy plant sap. The Detection of CMV coat protein with purified MAb-5. (A) Standard curve of the absorbance density of loaded purified CMV good linearity (1~100 ng/spot). (B) Absorbance of 2-µl of sSerial dilution of infected plant sap range 10^{-1}~10^{-5}. Each point represents the mean of three replicates.

ring with Pepo-CMV.

Quantification of the CMV in infected plants

The relationship between the absorbance density of immunostained spots and the amount (ranging from 1 to 100 ng per spot) of loaded purified Pepo-CMV gave linear standard curves (Figure 5A) Therefore, the absorbance values for serial dilution of infected plant sap of the same stain was straightforward to determine the real concentrations of the virus in infected plants (Figure 5B). As the dot-immunobinding assay uses only 2-µl aliquots, it is applicable to the determination of CMV concentrations higher than 128 ng/ml. This is less than in standard TAS-ELISA. However, from a practical point of view, the sensitivity of the dot-immunobinding assay is sufficient for the determination of CMV concentrations in infected plant sap.

DISCUSSION

A reliable plant pathogen diagnostic method requires highly sensitivity and specificity, especially when it is used for determining the sanitary status of plant materials to be vegetatively propagated. A variety of properties have been used to group CMV isolates into three subgroups: IA, IB, and II including antigenic specificity, symptomology, peptide mapping, and nucleic acid analyses (Anderson et al., 1995; Palukaitis and Zaitlin, 1997; Quemada et al., 1989; Roossinck et al., 1999). For the large-scale diagnosis of viral diseases and elucidating the antigenic structure of plant viruses at molecular level, monoclonal antibodies that are specific for plant viruses have been found very useful (Van Regenmortel, 1984). In addition to the diagnosis, MAbs were applied in identifyying the functional regions of the CMV RNA replicase. However, it is known that some plant viruses, including CMV are poor immunogens for preparation of antisera by conventional means (Palukaitis et al., 1992).

The production of MAbs and PAbs is an efficient means of using long-term maintenance to produce antibodies. However, while MAbs from ascites can yield high amounts and titers of antibody comparable to cell cultured supernatant. The fact that the reactions of these Japanese CMV isolates were serologically very homogenous with polyclonal antiserum accounts for why a MAb array consisting of MAb-I, II, and III, could be applied to identify CMV isolates according to seroreactive pattern. The results obtained from the Japanese CMV isolates revealed that there are a large number of the different epitopes on virions coat protein detected with MAbs (Table 1). These epitopes are inferred by the observation that subsets of the MAbs recognized the same isolates, usually with similar intensities of signals. In fact, the ability of MAb-M2-4 to cross react with every isolate of CMV tested in ELISA, DIBA, and even western blot indicates the presence of common epitope in the CP of CMV isolates (Hsu et al., 2000). This might be appropriate depending on the hypothesis that transgenically expressed antibodies or antibody fragments recognizing critical epitopes on structural or non-structural proteins of invading viruses may interfere with viral infection and confer viral resistance (Voss et al., 1995).

Analyses of antibody reactivity of a large number of isolates have been studied and identified CMV coat proteins appear to be different from the majority (Anonymous, 1998; Hsu et al., 2000). This indicates that these MAbs can be used reliably to type majority of CMV isolates to a subgroup. Readily, specifically, sensitivity, and quantitatively detected CMV with ELISA and DIBA

are assays that allowed detection of CMV in purified and infected plant. However, while DAS-ELISA allowed reliable CMV purified preparation detected with highly sensitivity nanogram level, DIBA detected less sensitivity than TAS-ELISA but can be prepared in the field and stored for long periods without loss reactivity. This possibility is very convenient for epidemiological studies in which large areas away from the laboratory are to be sampled in a short period. It also allows several membranes to be dotted with the same sample for future processing with new antibodies or membranes to be mailed to a different laboratory where these antibodies are available.

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